Characterization of leukotriene D_4-induced gene signatures in human endothelial cells

Dissertation

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Introduction

1.1. Leukotrienes and their receptors

Leukotrienes (LTs) are short-lived lipid mediators derived from arachidonic acid (AA) via the 5-lipoxygenase (5-LO) pathway. LTs were first described in the 1930s by Feldberg and Kellaway as the material released from guinea pig lung in response to antigen stimulus. This material was first called "slow reaction smooth muscle-stimulating substance" after its smooth muscle contracting capacity in guinea pig ileum and in other vascular preparations, then later renamed "slow-reacting substance of anaphylaxis" (SRS-A). The composition of SRS-A was identified in the 1970s [reviewed in Brink 2003].

1.1.1. LT biosynthesis

LTs are produced by certain leukocytes, mainly of myeloid origin: neutrophil, basophil and eosinophil granulocytes, mast cells, monocytes and macrophages. Since these cells constitutively express the components of the enzyme cascade necessary for LT synthesis and contain considerable amounts of esterified AA, they can generate and secrete LTs upon stimulation [Peters-Golden 2004]. Regarding their LT production profiles, myeloid cells show the following differences: neutrophils produce primarily LTB_4, and mast cells, basophils and eosinophils mainly cysteinyl LTs (cysLTs), whereas monocytes and macrophages produce both types [Peters-Golden 2004]. Other leukocytes such as tonsillar B lymphocytes and dendritic cells (DCs) are also capable of LT synthesis, though in smaller quantities [Jakobsson 1991, Spanbroek 2000].

LT synthesis is dependent on 5-LO. This enzyme, in concert with the 5-LO activating protein (FLAP), catalyzes the conversion of AA into LTA_4, which can be hydrolyzed into LTB_4 or further processed into cysLTs (Figure 1).

Stimuli that trigger increased intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_{ic}$) can activate the cytosolic phospholipase A_2 ($cPLA_2$), which is considered the most important cytosolic phospholipase providing substrate for 5-LO [Peters-Golden 2004]. After moving to the nuclear envelope, $cPLA_2$ liberates AA from membrane phospholipids, which is transferred to 5-LO via FLAP. 5-LO can

localize in both the cytoplasm and the nucleus [Brock 2005]. Upon increase in $[Ca^{2+}]_{ic}$, 5-LO moves to the cellular membranes and assembles with complexes of FLAP and LTC_4 synthase (LTC_4S). The membrane association and the catalytic activity of 5-LO may be regulated via phosphorylation of the enzyme, ATP binding or by glutathione peroxidases [Rådmark 2000, Brock 2005]. In the first step of LT synthesis, AA is oxygenated and forms 5-(*S*)-hydro-peroxyeicosatetraenoic acid (5-HPETE), which is subsequently dehydrated to LTA_4. LTA_4 is an unstable epoxide, which can react with water to form inactive metabolites, or it can serve as substrate for two enzymes. LTA_4 can be hydrolyzed into LTB_4 by LTA_4 hydrolase [Shimizu 1984, Rådmark 1984], or the membrane bound LTC_4S can conjugate LTA_4 with reduced glutathione, thus converting it into LTC_4, the parent compound of the cysLTs [Yoshimoto 1985, Penrose 1992]. LTC_4 is exported from the cell via an ATP-dependent step by a multidrug resistance-associated protein [Leier 1994]. Extracellularly, LTC_4 either binds to the cysteinyl LT receptors (cysLT-Rs) or it is further metabolized. γ-Glutamyl transpeptidase and γ-glutamyl leukotrienase convert LTC_4 into LTD_4 [Anderson 1982], which, in turn, can be cleaved by a dipeptidase into LTE_4 [Lee 1983]. LTD_4 and LTE_4 also bind to the cysLT-Rs.

A process termed *transcellular biosynthesis* or *transcellular metabolism*, enables LT production to proceed also in those cells that do not express 5-LO and, therefore, cannot initiate the LT cascade. Thus, LTA_4 can be released from inflammatory cells and is then taken up by nearby cells or platelets, which metabolize it into LTB_4 or LTC_4 [McGee 1986, Maclouf 1988]. Human umbilical vein endothelial cells (HUVECs) were shown to produce LTB_4 and cysLTs from both exogenously added and granulocyte-derived LTA_4 [Claesson 1988]. EC-derived LTs may act in a paracrine fashion on neighboring cells and also in an autocrine fashion on ECs. This raises the possibility that ECs can exacerbate inflammatory processes by synthesizing LTs.

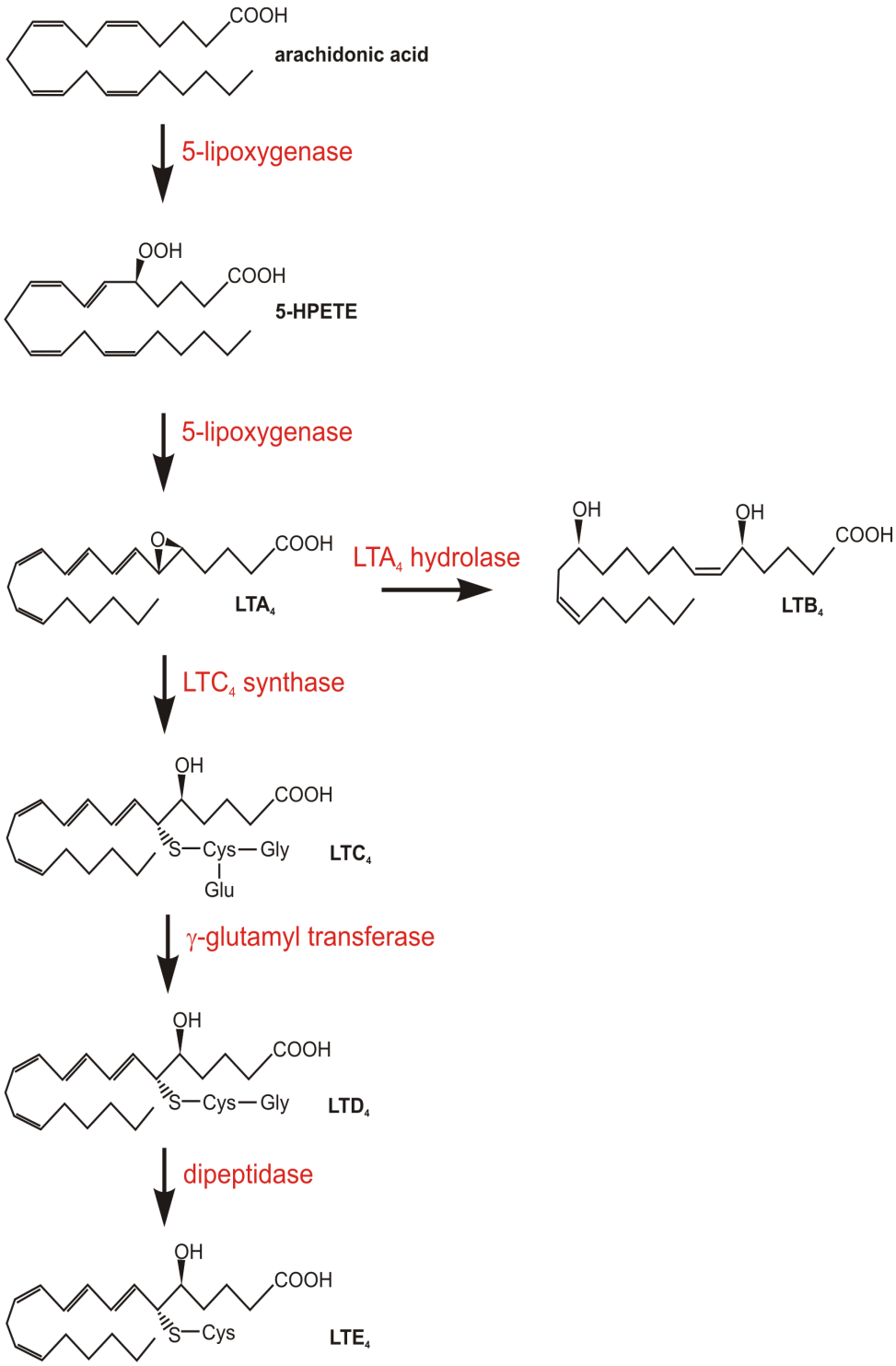

Figure 1. Enzymatic steps of LT biosynthesis. AA released from membrane phospholipids is converted into 5-(*S*)-hydroperoxyeicosatetraenoic acid (5-HPETE) and subsequently into LTA_4 by 5-LO. LTA_4 can be either hydrolyzed into LTB_4 by LTA_4 hydrolase or converted into LTC_4 via conjugation with glutathione by LTC_4 synthase. Removal of the glutamyl group of LTC_4 by γ-glutamyl transferase forms LTD_4, which can be further cleaved into LTE_4 by a dipeptidase.

1.1.2. Physiological and pathological role of the LTs

LTs are proinflammatory mediators produced by stimulated leukocytes at the site of antigen entry or tissue injury. LTs are best known for their vasoactive and smooth muscle contracting effects [reviewed in Brink 2003, Kanaoka 2004]. Although their involvement in innate and adaptive immune responses is increasingly investigated, there still remain a lot of open questions regarding their physiological and pathophysiological role(s). The effector functions influenced by LTs during the immune response might include effects on leukocytes (accumulation, cytokine generation, microbial killing, phagocytosis) and on the micro-environment (increased vascular permeability, smooth muscle contraction, broncho-constriction, enhanced mucus secretion) [reviewed in Funk 2001, Kanaoka 2004, Peters-Golden 2004]. These observations are based primarily on *in vitro* results. Within the last few years, several studies addressed and confirmed the role of LTs *in vivo* in mouse models, e.g. in alveolar clearance and phagocytosis during *Klebsiella pneumoniae* infection [Bailie 1996], and in DC migration [Robbiani 2000].

Altered LT generation has been associated with several human diseases. Decreased LT producing capacity was reported in immunosuppressed states due to malnutrition or drug treatment [reviewed in Peters-Golden 2004]. Peripheral blood neutrophils and monocytes from HIV infected patients showed impaired LTB_4 generation, which was reversed by *in vivo* treatment with granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage CSF (GM-CSF) [Coffey 1998, Coffey 1999]. Increased levels of LTs were observed in humans during viral and bacterial infections [reviewed in Peters-Golden 2004], and in patients with asthma exacerbations [reviewed in Kanaoka 2004]. Involvement of cysLTs in airway inflammation is supported by studies of mouse models of asthma [reviewed in Kanaoka 2004], by effects of exogenously administered LTs resembling symptoms of airway inflammatory diseases [reviewed in Brink 2003], and by reports on beneficial effects of LT modifier drugs in respiratory disorders [reviewed in Busse 2005]. Nonetheless, the role they play in disease states still remains to be clarified.

1.1.3. LT receptors

LTs act through G protein-coupled seven transmembrane domain containing receptors (GPCRs). The four known LT receptors (LT-Rs) belong to two main classes: BLT-Rs, which bind LTB_4 and other eicosanoids, and cysLT-Rs, which bind LTC_4, LTD_4 and LTE_4.

1.1.3.1. BLT-Rs

BLT_1-R was the first LT-R to be identified [Yokomizo 1997]. It is predominantly expressed by leukocytes [Yokomizo 2001], but endothelial and vascular smooth muscle cells were also shown to express it [Lötzer 2004]. By contrast, BLT_2-R is widely expressed, most strongly in the spleen, liver and ovary, and it was also detected in leukocytes and endothelial and vascular smooth muscle cells. In contrast to BLT_1-R, which binds LTB_4 with high affinity, BLT_2-R has lower affinity to LTB_4, and eicosanoids with LTB_4-related structure can also activate this receptor in a dose-dependent manner [Yokomizo 2001]. According to phylogenetic analyses, the BLT-Rs have a closer relationship to chemoattractant receptors, e.g. to the complement fragment receptors C5aR and C3aR, and to the formyl peptide receptor 1 (FPR1), than to the cysLT-Rs [Izumi 2002]. Both BLT-Rs are expressed in human atherosclerotic lesions [Spanbroek 2003], but their transcript levels are not higher than in control tissues [Qiu 2006a]. The data of Qiu *et al.* (2006a) suggest elevated LTB_4 production within atherosclerotic plaques, which implicates BLT-Rs in atherogenesis, but the cell types expressing BLT-Rs and the effects of BLT-R stimulation in these cells remain to be identified.

1.1.3.2. CysLT-Rs

Pharmacological studies applying various inhibitors and radioligand binding assays indicate that there are at least two human cysLT-Rs [reviewed in Evans 2002, Brink 2003, Capra 2004]. Both receptors were identified by ligand fishing approaches screening orphan GPCRs. $CysLT_1$-R was cloned by two groups independently [Lynch 1999, Sarau 1999]. This gene is localized on chromosome Xq13.2 and the encoded protein consists of 337 amino acids. $CysLT_1$-R was shown to be highly expressed in peripheral blood leukocytes and in spleen, and at a lower level in several other tissues, e.g. pancreas, placenta, colon, kidney, liver, small intestine, brain and lung [Sarau 1999, Lynch 1999]. In the lung, $cysLT_1$-R expression was localized in macrophages and airway smooth muscle cells [Lynch 1999, Figueroa 2001].

The human $cysLT_2$-R was cloned by three groups [Heise 2000, Takasaki 2000, Nothacker 2000]. The CYSLTR2 gene was mapped to the chromosome 13q14.12 and encodes a protein of 346 amino acids. Northern blot analysis showed strong expression of the $cysLT_2$-R mRNA in the heart, adrenal gland and placenta, and moderate levels were found in several other tissues, e.g. spleen, lymph nodes, peripheral blood leukocytes and throughout the central nervous system [Heise 2000, Nothacker 2000, Takasaki 2000]. Heise *et al.* (2000) provided circumstantial evidence for $cysLT_2$-R expression in lung interstitial macrophages and smooth

muscle cells by *in situ* hybridization. By contrast, Takasaki *et al.* (2000) could not confirm the presence of cysLT$_2$-R mRNA in lung by Northern blotting from tissue lysates. In partially purified blood leukocyte populations, cysLT$_2$-R mRNA was detected in monocytes and eosinophil granulocytes [Heise 2000]. The distinct expression pattern of the two cysLT-Rs suggests different functions.

The cysLT$_1$-R and cysLT$_2$-R proteins show only 38% identity to each other at the amino acid level [Heise 2000]. According to phylogenetic analyses, they belong to the rhodopsin subfamily of the GPCR superfamily [reviewed in Izumi, 2002; Capra, 2004]. Among others, they are closely related to purinergic or pyrimidinergic receptors and thrombin receptors [Capra 2004]. This close relationship is supported by the fact that cysLT-Rs can be activated by uridine diphosphate [Mellor 2002].

The two cysLT-Rs differ from each other in their sensitivity toward cysLT$_1$-R selective antagonists and in ligand binding affinity. In cysLT$_1$-R transfected cells cysLTs induced a dose-dependent increase in $[Ca^{2+}]_{ic}$ with the rank order $LTD_4 >> LTC_4 > LTE_4$. The cysLT-induced mobilization of intracellular Ca^{2+} was inhibited by Montelukast, Zafirlukast, Pranlukast, Pobilukast, MK571 as well as by Bay u9773 [Lynch 1999, Sarau 1999]. The cysLT$_2$-R-transfected cells also responded to cysLT-stimulation with an elevated $[Ca^{2+}]_{ic}$ in a dose-dependent manner. The affinity of cysLT$_2$-R towards the agonists is $LTD_4 = LTC_4 > LTE_4$ [Heise 2000, Nothacker 2000, Takasaki 2000]. The cysLT$_1$-R inhibitors were ineffective

	cysLT$_1$-R	***cysLT$_2$-R***
ligands	$LTD_4 >> LTC_4 > LTE_4$	$LTD_4 = LTC_4 > LTE_4$
antagonists	Montelukast, Zafirlukast, Pranlukast, Pobilukast, MK571, Bay u9773	Bay u9773 (partial agonist)
expression	high: PBL and spleen lower: pancreas, placenta, colon, kidney, liver, brain, lung	high: heart, adrenal gland, placenta lower: spleen, PBL, CNS
inducing cytokines	IL-4, IL-13, IFNγ, TGFβ	IL-4, IFNγ
G protein-coupling	G_i, G_q, G_o?	G_i, G_q, G_o?
Ca^{2+}-response	solid peak	oscillation

Table 1. Comparison of cysLT$_1$-R and cysLT$_2$-R regarding their ligand affinity, selected antagonists, expression and G protein utilization. On the basis of Brink *et al.* (2003), Espinosa *et al.* (2003), Fujii *et al.* (2005), Heise *et al.* (2000), Lynch *et al.* (1999), Lötzer *et al.* (2003), Thivierge *et al.* (2001). PBL: peripheral blood leukocytes, CNS: central nervous system

[Heise 2000, Nothacker 2000, Takasaki 2000], and the dual cysLT$_1$-R / cysLT$_2$-R inhibitor Bay u9773 also acted as a partial agonist at the cysLT$_2$-R by eliciting a concentration-dependent increase in $[Ca^{2+}]_{ic}$ [Nothacker 2000] (Table 1).

Recently, an orphan GPCR, GPR17, has been identified as a third putative cysLT-R [Ciana 2006]. GPR17 is characterized as a dual receptor for uracil nucleotides and cysLTs. In transfected cells GPR17 responds to cysLTs in the same concentration range as the two other cysLT-Rs, but shows different ligand binding affinity: LTC$_4$>>LTD$_4$. GPR17 is inhibited by the cysLT$_1$-R antagonists Montelukast and Pranlukast. Expression of GPR17 was detected by semi-quantitative PCR in brain, kidney, heart and HUVECs [Ciana 2006].

Pharmacologic profiling studies of human pulmonary artery smooth muscle provided indications that a further cysLT-R or a new subtype may exist. Stimulation with LTC$_4$ or LTD$_4$, but not with LTE$_4$, induced contractions in human pulmonary artery preparations. Neither the classical cysLT$_1$-R antagonists, nor the cysLT$_1$-R / cysLT$_2$-R dual antagonist could modify the contractions. However, cell type-specific expression and activation of the receptors, or effects resulting from concomitant stimulation of endothelial and smooth muscle cell cysLT-Rs may explain these observations [Walch 2002].

1.1.3.3. Functional studies on cysLT-Rs

CysLT$_1$-R expression and function have been extensively studied in various organ preparations and *in vitro* systems [Espinosa 2003, Mellor 2001, Thivierge 2001 and reviewed in Brink 2003 and Capra 2004]. However, there is very little information available on the cysLT$_2$-R. Experiments with cord blood-derived mast cells that express both cysLT-Rs indicate distinct roles and signaling pathways for the two receptors [Mellor 2001, 2002 and 2003]. In these cells cysLT$_2$-R was responsible for cysLT-induced interleukin 8 (IL-8) production, and it was partially involved in IL-5 secretion. Since both receptors are present on mast cells, downstream synergism between cysLT$_1$-R and cysLT$_2$-R cannot be excluded. In transient transfectants cysLT$_2$-R did not couple to G_i, but induced Ca^{2+} signals probably via G_q activation [Heise 2000]. By contrast, in mast cells cysLT$_2$-R coupling to G_i was observed, and the cysLT-triggered Ca^{2+} responses were entirely dependent on cysLT$_1$-R [Mellor 2003].

Analyses of $[Ca^{2+}]_{ic}$ elevations by the two cysLT-Rs in non-transfectant systems where one of the receptors was only marginally present revealed differences in kinetics, amplitude, duration and oscillatory patterns [Lötzer 2003, Sjöström 2003]. LTD_4 challenge of the $cysLT_1$-R in MonoMac-6 cells (human monocytic cell line) that express mainly the $cysLT_1$-R induced a rapid, short-lived response, whereas stimulation of the $cysLT_2$-R on HUVECs resulted in oscillations maintained for >60 minutes [Lötzer 2003].

$CysLT_1$-R mRNA induction by IL-4, IL-13, interferon γ (IFNγ) or transforming growth factor β (TGFβ) was described in various cell types [Thivierge 2001, Mellor 2001, Espinosa 2003]. On the other hand, augmented $cysLT_2$-R transcription was observed only in response to IL-4 [Lötzer 2003, Mellor 2003, Sjöström 2003]. In HUVECs other inflammatory mediators such as lipopolysaccharide (LPS), tumor necrosis factor α (TNFα) and IL-1 did not alter $cysLT_2$-R expression [Lötzer 2003, Sjöström 2003]. Peripheral blood eosinophils express both cysLT-Rs, and in asthmatic patients expression levels on the cell surface are significantly enhanced, but their functions were not further characterized [Fujii 2005].

These data indicate that regulation of cysLT-R expression, G protein-coupling and signaling depend on cell type and pathophysiological conditions. Table 1 provides a short summary on $cysLT_1$-R and $cysLT_2$-R attributes.

1.1.3.4. CysLT-Rs in the cardiovascular system

LTs have several effects on the cardiovascular system, e.g. on blood pressure regulation, coronary artery contraction, oedema formation [reviewed in Lötzer 2005]. Kamohara *et al.* examined the distribution of cysLT-Rs in the heart by *in situ* hybridization and RT-PCR. $CysLT_1$-R was expressed in the pericardium at a low level. However, $cysLT_2$-R was abundantly expressed in the left and right atria and the left ventricle and showed weaker expression in the artery, where mRNA was detected only in smooth muscle cells and not in the endothelium. In cultured coronary artery smooth muscle cells, LTC_4 increased the $[Ca^{2+}]_{ic}$, and this increase was not inhibited by $cysLT_1$-R antagonists. LTC_4 also initiated migration of these cells [Kamohara 2001]. CysLT-R expression was also determined and confirmed in atherosclerotic lesions [Spanbroek 2003], but neither receptor showed increased mRNA levels compared to iliac artery samples as control [Qiu 2006a]. The presence of cysLT-Rs in the diseased vessel wall may implicate them in atherogenesis, but these studies provide data only about the mRNA levels of these receptors; their expression pattern and function in the lesion remain to be identified.

The role of $cysLT_2$-R in the vascular system was investigated *in vivo* in transgenic mice expressing the human $cysLT_2$-R in endothelial cells [Hui 2004]. Both endogenous cysLTs elicited by passive cutaneous anaphylaxis and exogenously added LTC_4 caused enhanced vascular permeability in the ear vasculature of transgenic mice. The regulatory role of $cysLT_2$-R on vascular permeability is supported by data of Beller *et al.* (2004), who found reduced leakage in $cysLT_2$-$R^{-/-}$ mice. As a systemic effect, the pressor effect induced by LTC_4 administration was abolished in $cysLT_2$-R transgenic mice [Hui 2004]. This may be the result of increased NO production after LTC_4 injection, though the changes in blood pressure and NO levels show divergence in time. The source of NO was not identified either, which leaves the possibility of indirect regulation open. Lung endothelial cells prepared from the transgenic mice showed stronger response to LTC_4 and LTD_4 in $[Ca^{2+}]_{ic}$ increase than those from control mice, confirming the presence of functional cysLT-Rs. In the ECs from transgenic mice, however, the LTC_4- and LTD_4-triggered signals differed both in magnitude and kinetics, which contradicts the data obtained in human systems, where LTC_4 and LTD_4 are equipotent agonists of the $cysLT_2$-R [Heise 2000, Evans 2002, Lötzer 2003]. This shows that, though such studies are important, their results cannot always be transferred directly and need to be confirmed in human systems.

1.1.4. LT effects on the endothelium

The endothelium lining the blood vessels forms a barrier between the blood and the vessel wall. Due to this location, ECs can be exposed to LTs derived both from blood leukocytes and inflammatory leukocytes localized in the altered vessel wall. The effects of LTs on the endothelium is mostly studied *in vitro* using HUVECs.

As a chemotactic mediator, LTB_4 enhances leukocyte attachment to the endothelium [Heimbürger 1995, Yokomizo 1997, Tager 2003]. This effect was considered to be the result of BLT-R activation on the leukocytes. It has recently been described that HUVECs express both BLT-Rs [Lötzer 2003], and BLT-R expression can be induced by inflammatory mediators such as TNFα, LPS and IL-1β, and by LTB_4 itself [Qiu 2006b]. HUVECs respond to LTB_4 stimulation with increased $[Ca^{2+}]_{ic}$, NO generation and monocyte chemoattractant protein 1 (MCP-1) release [Qiu 2006b]. These data show that LTB_4 acts on both partners to promote leukocyte-EC interaction.

CysLTs have diverse post-transcriptional effects on HUVECs, including the release of AA and its derivatives as well as the expression of adhesion molecules.

LTC_4 and LTD_4 induce release of AA, prostacyclin (PGI_2), prostaglandin $F_{2\alpha}$ [Cramer 1983] as well as platelet-activating factor (PAF) [McIntyre 1986] in a dose-dependent manner. In contrast to the transient and rapid PGI_2 and PAF release mediated by other vasoactive agonists, cysLTs trigger continuous liberation of AA and its derivatives [Cramer 1983, McIntyre 1986]. The two major phospholipid-derived products, PGI_2 and PAF, have opposite effects on polymorphonuclear cells (PMNs). Upon vascular injury, PAF synthesized by ECs can activate platelets and attract PMNs to promote hemostasis. At the same time, PGI_2 released by ECs can control hemostasis by limiting the number of involved platelets and PMNs and by localizing thrombus formation.

In addition to liberation of the phospholipid-derived mediators, cysLTs can mobilize the secretory granules called Weibel-Palade bodies, thus promoting rapid von Willebrand factor (vWF) secretion and P-selectin surface expression [Datta 1995]. The mechanism involved in this secretory process is not fully understood. A cysLT-induced $[Ca^{2+}]_{ic}$ increase was shown to be necessary for vWF secretion, but the Ca^{2+}-activated protein kinase C (PKC) is not the primary mediator. vWF release could be inhibited with high concentrations (5 and 10 µM) of the $cysLT_1$-R-specific antagonist Pobilukast. By contrast, Pedersen *et al.* found that three structurally unrelated $cysLT_1$-R antagonists (Zafirlukast, Pranlukast and Pobilukast; highest concentration 1 µM) did not have any effects on LTC_4- and LTD_4-induced P-selectin expression. At 1 µM and 100 nM concentrations, LTD_4 was less effective than LTC_4. LTC_4 was similarly effective as histamine, a well-known endothelial P-selectin inducer [Pedersen 1997]. These controversial findings are not necessarily contradictory. Datta *et al.* did not investigate the effect of $cysLT_1$-R antagonists on P-selectin expression, and Pedersen *et al.* did not measure vWF secretion or show involvement of Weibel-Palade bodies. Both P-selectin and vWF can be expressed and released independently from the Weibel-Palade bodies. Moreover, the content of Weibel-Palade bodies was shown to change dynamically depending on micro-environmental conditions and stimuli, and the existence of different subsets with various contents was also revealed [reviewed in Rondaij 2006].

It has to be noted that the cysLT-Rs had not yet been cloned and characterized at that time. In addition, data on cysLT-R expression on HUVECs are also inconsistent and may depend on

cell culture conditions [Sjöström 2001, Lötzer 2003]. For PAF and PGI_2 synthesis, it was not clarified which receptor(s) are responsible for the effect.

1.2. Thrombin and its receptors

Thrombin, a trypsin-like serine protease, is the main protease in the blood coagulation system. Activation of both arms of the coagulation cascade, namely the intrinsic (or contact factor) pathway and the extrinsic (or tissue factor) pathway, converge on thrombin. Once generated, thrombin catalyzes the final steps to clot formation, i.e. the conversion of fibrinogen to fibrin and the activation of factor XIII (FXIII) and platelets (Fig. 2, 3). Besides its direct effector role in clot formation, thrombin initiates several positive and negative feedback mechanisms regulating the coagulation process (Fig. 3). In addition, thrombin elicits numerous cellular effects via its receptors, the so called protease-activated receptors (PARs).

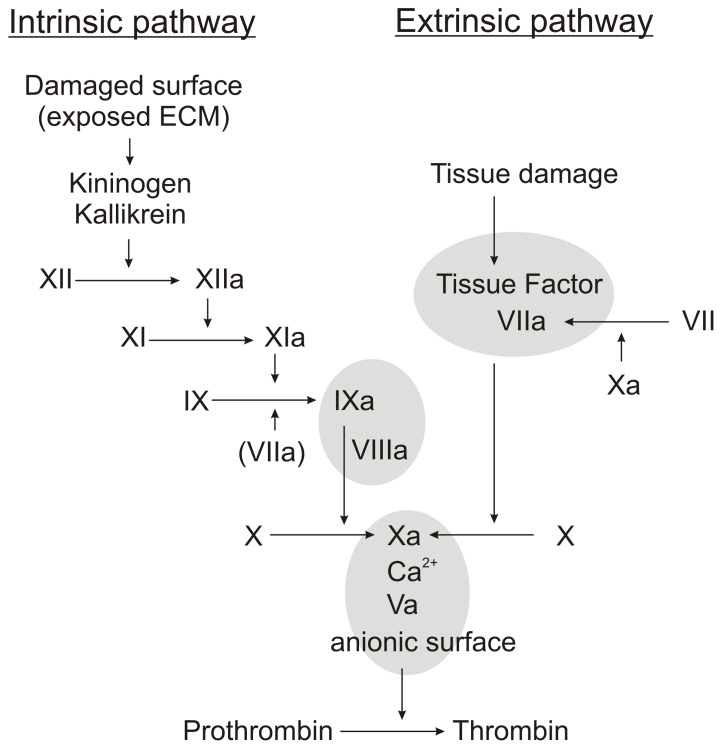

Figure 2. Schematic view of the coagulation cascade leading to thrombin formation. The intrinsic pathway is initiated through contact and activation of FXII by abnormal surfaces caused by injury. Tissue damage can trigger TF expression in ECs or expose TF-bearing vascular wall cells, which induces the extrinsic pathway. Activated factors are marked with "a". Gray ovals indicate proteinase complexes, where TF, and factors Va and VIIIa do not have enzymatic activity. ECM: extracellular matrix. [After Berg 2001]

1.2.1. Thrombin generation and inactivation

Thrombin is derived from prothrombin, its inactive precursor, which is synthesized in the liver and is secreted into circulation. Upon vascular injury, activation of upstream coagulation factors results in formation of the prothrombinase complex consisting of FVa, FXa, Ca^{2+}, and anionic phospholipids on the surface of platelets or cells (Fig. 2). Prothrombin is localized to membrane surfaces via several domains, which compose the F1+2 fragment after cleavage by FXa. Loss of the F1+2 module enables thrombin to freely diffuse and to activate its substrates, and exposes functional regions important for thrombin activity and specificity [Crawley 2006, Davie 2006].

To prevent inappropriate clot formation or bleeding, thrombin generation and activity are tightly controlled by several mechanisms. Thrombin itself regulates its own generation by activating factors V, VIII and XI, but also its own termination via the protein C system in the case of normal endothelium. Due to the great amount of circulating serine protease inhibitors, active thrombin is short-lived in the blood. The most important endogenous inhibitors are antithrombin, heparin cofactor II, protease nexin I, which all inhibit the catalytic activity of thrombin. Exogenous thrombin inhibitors such as hirudin, hemadin and rhodniin isolated from

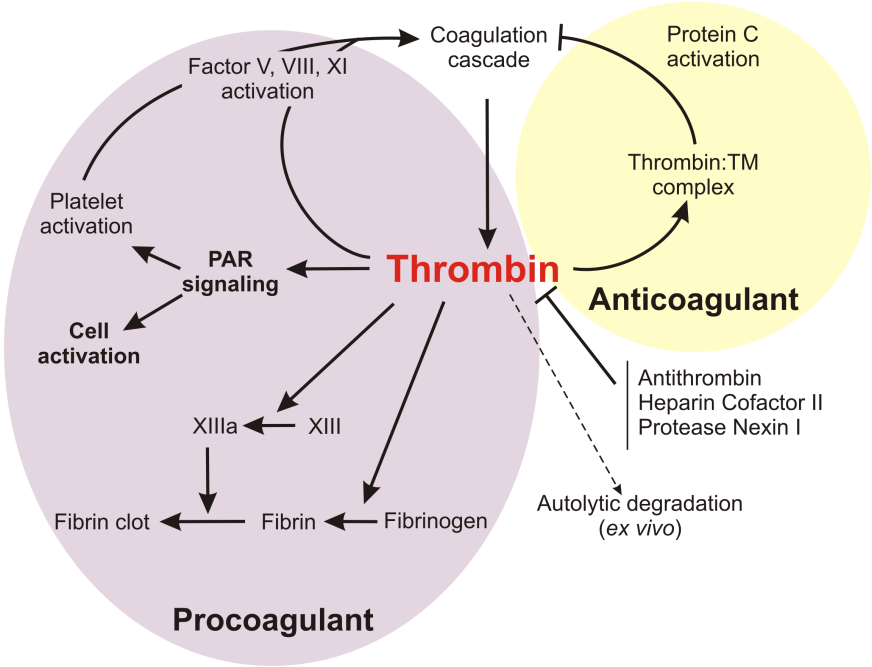

Figure 3. The central role of thrombin in hemostasis. Activation of the coagulation cascade leads to thrombin formation. Besides its essential role in clot formation, thrombin triggers its own production by activating platelets and factors V, VII and XI, and induces cellular responses via its receptors. Thrombin binding to thrombomodulin (TM) activates the protein C system, which negatively regulates the coagulation cascade. Serine protease inhibitors and possible degradation lead to inactivation of thrombin. [After Crawley 2006]

hematophagous animals have similar inhibitory properties [Davie 2006]. In addition, autolytic degradation of thrombin was also shown, but based on present knowledge it only takes place *ex vivo* after prolonged standing in solution (Fig. 3) [Davie 2006].

1.2.2. Thrombin receptors

Thrombin signaling is mediated through members of the protease-activated receptor (PAR) family. Thrombin can activate three of the four known receptors. PARs are GPCRs expressed on a variety of cell types and can be activated by several proteases that are usually generated during inflammation and injury (Table 2). Thus, actions mediated by PARs contribute to hemostasis, inflammation and nociception.

1.2.2.1. Mechanism of PAR activation and signaling

Proteases activate PARs by a mechanism unique among GPCRs but common for PARs. Cleaving the receptor unmasks a new N-terminal domain, which serves as a tethered peptide ligand and binds irreversibly to the body of the receptor. This intramolecular ligation induces conformational changes resulting in G protein activation. Principally, liberating the tethered ligand by any protease activates the receptor and triggers cellular effects. The specificity of the

Receptor	*Activating protease*	*Expression in ECs*	*Expression in other cells*
PAR-1	thrombin trypsin FXa APC	HUVECs, HAECs, HCAECs, HMECs, rat sinusoidal ECs, porcine CAECs	platelets, fibroblasts, neurons, T cells, VSMCs, rat cardiomyocytes
PAR-2	trypsin mast cell tryptase TF/FVIIa/FXa complex APC	HUVECs, HAECs, HMECs	fibroblasts, neurons, neutrophil granulocytes, epithelial cells, bovine VSMCs, rat cardiomyocytes
PAR-3	thrombin trypsin	human and rat brain MECs, HUVECs(?)	VSMCs, mouse platelets
PAR-4	thrombin trypsin neutrophil cathepsin G	rat aortic ECs, stimulated HCAECs	human platelets, VSMCs, rat cardiomyocytes

Table 2. Comparison of PARs regarding their activating enzyme and expression in the vasculature. On the basis of Bretschneider (1999, 2000 and 2003), Bunnett (2006), Coughlin (2005), Macfarlane (2001), Minami (2004b), Ossovskaya (2004) and Sabri (2000 and 2003). HAECs: human aortic ECs, HCEAECs: human coronary artery ECs, HMECs: human microvascular ECs, VSMCs: vascular smooth muscle cells

reaction lies in differences in the proteases, in the receptors (both the ligand and the ligand binding site) and in the cell types expressing them. Peptides corresponding to the protein sequence of the activating site cause receptor activation without proteolysis [reviewed in Ossovskaya 2004, Coughlin 2005, Davie 2006]. PARs couple to several subtypes of G proteins and can initiate a signaling network in a cell type-dependent fashion [reviewed in Macfarlane 2001, Ossovskaya 2004, Coughlin 2005]. Termination of the signaling of irreversibly activated receptors is not clearly understood. C-terminal phosphorylation by G protein-coupled receptor kinases and PKC, and interaction with β-arrestin and other adaptor(s) lead to desensitization and internalization of PAR-1 [reviewed in Macfarlane 2001, Ossovskaya 2004]. In contrast to reversibly activated GPCRs, which recycle to the surface after internalization, a large proportion of PAR-1 is sorted to lysosomes and becomes degraded [Trejo 2003]. Thrombin responsiveness is maintained by an intracellular pool of presynthesized, naïve receptors, which, upon stimuli, translocate to the plasma membrane in a cell type-dependent manner, and by newly synthesized receptors [Macfarlane 2001].

1.2.2.2. PARs in the cardiovascular system

PARs are expressed on many cell types of the vascular system (ECs, vascular smooth muscle cells [VSMCs], fibroblasts, neurons, cardiomyocytes) and by cells contributing to distinct pathophysiological conditions (platelets, monocytes, T cells, mast cells, neutrophils). In addition to PAR-1, which is present on nearly all of these cells, several cell types express other PAR(s) in various combinations (shown at least *in vitro*) (Table 2). The receptors differ in availability, affinity, activating mechanism, and thus, they are activated under different microenvironmental conditions and mediate distinct effects. Vascular cells that are separated from blood flow (VSMCs, fibroblasts, cardiomyocytes) constitutively express tissue factor (TF). Those in contact with blood (ECs and monocytes) do not express TF under physiological conditions, but TF is induced in response to tissue damage, inflammation or coagulation proteases [Mackman 2004, Stenina 2003].

Upon vascular injury, thrombin is generated at high local concentrations on the surface of TF expressing cells and can activate these cells via their PARs. Under normal conditions PARs contribute to hemostasis and physiological blood clotting in response to injury. In cases of imbalanced hemostasis, chronic inflammation or sepsis, generation of thrombin and other proteases is accelerated, which leads to enhanced activation of PAR-expressing cells. These, in turn, maintain their thrombogenic and inflammatory phenotype. This can result in thrombus formation and development of vascular disorders. PARs are implicated in mechanisms of

several vascular diseases, e.g. atherosclerosis, coronary artery syndromes, cardiac infarction and stroke [reviewed in Mackman 2003, Ossovskaya 2004, Coughlin 2005, Bunnett 2006]. As the use of thrombin inhibitors may cause to excessive bleeding, PARs represent potential therapeutic targets in these diseases.

1.2.3. Thrombin effects on the endothelium

Thrombin triggered signaling in cultured ECs can lead to shape change, NO production, increased permeability [reviewed in Minami 2004b]. Similar to cysLTs, thrombin induces PGI_2 release [Weksler 1978], vWF secretion [Mayadas 1989], P-selectin expression [Sugama 1992], PAF synthesis [Prescott 1984]. Transcriptional changes result in a proinflammatory and prothrombotic phenotype via chemokine and growth factor secretion, increased expression of adhesion molecules and of TF. Thrombin also induces proliferation and migration of ECs, thus exerting angiogenic effects [reviewed in Coughlin 2000, Macfarlane 2001, Minami 2004b, Coughlin 2005].

In ECs PAR-1 is the major thrombin receptor [O'Brien 2000, Coughlin 2005]. ECs also express PAR-2, which was shown to be transactivated by PAR-1 in the presence of PAR-1 antagonist *in vitro* [O'Brien 2000]. In human microvascular ECs PAR-1 couples to G_q, $G_{i/o}$ and $G_{12/13}$ as demonstrated by inhibition of thrombin signaling with "minigenes" encoding a C-terminal peptide of the Gα subunits [Gilchrist 2001, Vanhauwe 2002]. Thrombin activates a complex net of signaling pathways, which involve the transactivation of the epidermal growth factor receptor (EGFR) and include the mitogen activated protein (MAP) kinase cascade, PI3 kinase, Rho kinase, PKC, other Ca^{2+}-regulated kinases and phosphatases, and ion channels [reviewed in Ossovskaya 2004, Coughlin 2005]. Thrombin-induced gene expression patterns and transcriptional regulatory mechanisms have been studied only recently [reviewed in Stenina 2003, Minami 2004b].

Rationale

Several vascular diseases are associated with inflammation. Leukocytes that accumulate at sites of vascular inflammation produce cytokines, growth factors and vasoactive molecules, among them LTs. Previously described effects of cysLTs in ECs include PAF release, vWF secretion, P-selectin surface expression, and oscillation in $[Ca^{2+}]_{ic}$, but the transcriptional changes induced by LTs remain to be determined. CysLTs act through three receptors, namely $cysLT_1$-R, $cysLT_2$-R, both of which are expressed in the vasculature, and a recently described cysLT-R called GPR17. Considerable amounts of data have been published on the $cysLT_1$-R, whereas the $cysLT_2$-R is less characterized. Since HUVECs selectively and constitutively express the $cysLT_2$-R, they are the first model to characterize $cysLT_2$-R functions in a non-transfectant primary cell system. Thrombin was chosen as positive control because LTD_4 and thrombin have several immediate post-transcriptional effects on the endothelium in common. Furthermore, both agonists act through GPCRs, and thrombin-activated HUVECs are a well characterized model in vascular biology and pathobiology.

Aims

The goal of this study was to characterize cysLT-induced gene signatures in endothelial cells. To this end, gene expression patterns in response to LTD_4 were analyzed in HUVECs at various time points.

In order to understand the magnitude of cysLT-mediated transcriptional changes, LTD_4-triggered gene signatures were compared to those induced by thrombin, a prototype vasoactive agonist of ECs.

Selected LTD_4-regulated genes, which have been implicated in vascular biology, were further studied at the protein level.

Materials and methods

2.1. Materials

All materials used in this work were from Invitrogen (Karlsruhe, Germany) unless otherwise noted.

2.1.1. Reagents

Cycloheximide (Sigma-Aldrich, Munich, Germany)

Cyclosporin A

LTD_4 (Cayman Chemical, Hamburg, Germany)

Montelukast (a gift from R. N. Young, Merck Frosst, Quebec, Canada)

Thrombin (Sigma-Aldrich)

TNFα (R&D Systems, Wiesbaden, Germany)

Vascular endothelial growth factor (VEGF; Cayman Chemical)

2.1.2. Kits

Actichrome TF Kit (American Diagnostica, Pfungstadt, Germany)

BD OptEIA Human IL-8 ELISA Kit II (BD Biosciences, Heidelberg, Germany)

Cloned AMV First-Strand cDNA Synthesis Kit

MSB Spin PCRapace Purification Kit (Invitek, Berlin, Germany)

Platinum Taq Polymerase Kit

RNeasy MicroKit and RNeasy MiniKit (Qiagen, Hilden, Germany)

2.1.3. Buffers and solutions

Phosphate buffered saline (PBS; without Ca^{2+} and Mg^{2+}) and Hank's balanced salt solution (HBSS; with Ca^{2+} and Mg^{2+}) were purchased from Invitrogen. For preparing the following buffers and solutions all materials were from Sigma-Aldrich unless otherwise noted.

10x stop solution: 0.05w/v% bromphenolblue, 48% glycerine, 0.1% SDS, 0.1 M EDTA

5x Laemmli buffer (pH=6.8): 2.5 M Glycerol, 0.3 M Tris, 3 M EDTA, 0.2 M SDS, 0.2 M β-mercaptoethanol, 1 mM bromophenol blue

Agarose gel: 2w/v% agarose (Roche Diagnostics, Mannheim, Germany), 50 ng/ml ethidium bromide in TAE buffer (see below)

Amidoblack destaining solution: 90v/v% methanol, 2v/v% acetic acid

Amidoblack staining solution: 0.1w/v% amidoblack, 45v/v% methanol, 10v/v% acetic acid

Blocking solution: 4w/v% non fat dry milk, 1w/v% BSA, 0.1v/v% Tween-20 in PBS

Calcium buffer (pH=7.4): 145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1.5 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, 0.25w/v% human serum albumin

FACS buffer: 2 mM EDTA, 1% fetal calf serum (FCS) in PBS

Ponceau Red staining solution: 0.5w/v% Ponceau S, 1v/v% acetic acid

Resolving gel (10%): 33% Acrylamide/Bis Solution (30% T, 2.67% C; Roth, Karlsruhe, Germany), 375 mM Tris (pH=8.8), 0.1w/v% SDS, 2.7 mM TEMED, 2.2 mM APS

Running buffer (pH=8.3): Rotiphorese (Roth) diluted in 1:10

Stacking gel (4%): 13% Acrylamide/Bis Solution (30% T, 2.67% C), 125 mM Tris (pH=6.8), 0.1w/v% SDS, 2.7 mM TEMED, 2.2 mM APS

TAE buffer (pH=8.0): 40 mM Tris, 20 mM glacial acetic acid, 1 mM Na_2EDTA

TF lysis buffer (pH=7.4): 50 mM Tris, 100 mM NaCl, 0.1% Triton X-100

Transfer buffer (pH=8.3): 25 mM Tris, 192 mM glycine, 20v/v% methanol

Tris buffer (pH=7.5): 10 mM Tris, 10mM $MgCl_2$, protease inhibitor cocktail (Complete; Roche Diagnostics)

Tris-Triton buffer (pH=7.5): 10 mM Tris, 10 mM $MgCl_2$, protease inhibitor cocktail, 1% Triton X-100

Tween-PBS: 0.1v/v% Tween-20 in PBS

2.2. Methods

2.2.1. Endothelial cell isolation and culture

Endothelial cells (ECs) were isolated from human umbilical cord vein by collagenase digestion according to the protocol described by Jaffe *et al.* (1973), with minor modifications. Umbilical cords not older than 24 h were used. Cords were rinsed and stored in cold PBS until processing. 0.1% collagenase (collagenase type 2; Worthington, Lakewood, NJ) in HBSS was freshly prepared and sterile filtered. Collagenase amount was calculated from the cord length: ~1 ml / 1 cm. Petri dishes were preincubated for 30 min with endothelial cell serum-free medium (EC-SFM) containing 10 U/ml penicillin, 10 µg/ml streptomycin, 20 µg/ml fibronectin (FN) and 5% FCS:

cord length [cm]	dish [cm^2]	medium [ml]
20-45	21	5
>45	58	12

The vein was canulated with a blunt needle and perfused with warm HBSS to remove blood. The distal end was clamped; the vein was filled with 0.1% collagenase; then this end was also clamped shut. The cord suspended by the clamped ends was incubated in water bath for 3 min at 37°C. The effluent solution containing the endothelial cells was collected in a 50 ml tube and the vein was flushed with medium M199 containing 5% FCS (M199-FCS). Cells were centrifuged at 200 *g* for 5 min at 20°C, then resuspended and plated in the preincubated dish. About 5 h after isolation the dishes were washed with warm HBSS to remove non-adherent cells (mainly blood cells) and fresh medium (EC-SFM containing 20 ng/ml basic fibroblast growth factor [bFGF] and 10 ng/ml epidermal growth factor [EGF]) was added. Cells were cultured in a humidified incubator at 37°C with a 5% CO_2 atmosphere. Cells were fed every 2-3 days by replacing half of the medium with fresh EC-SFM containing 40 ng/ml bFGF and 20 ng/ml EGF.

After reaching optical confluence, cells were passaged. Medium was removed and the cells were gently rinsed with trypsin-EDTA. 2 ml trypsin-EDTA was added and cells were monitored under the microscope. The reaction was stopped with warm M199-FCS and the cells were transferred into a 50 ml tube. The dish was rinsed with M199-FCS and the cells were centrifuged at 200 *g* for 5 min at 20°C. The cell pellet was resuspended in EC-SFM and the cells were counted using a Coulter Counter. Cells were seeded at a density of 15,000-20,000

cells/cm^2 in EC-SFM containing 10 µg/ml FN, 20 ng/ml bFGF and 10 ng/ml EGF. HUVECs were used in the first two passages.

Cells were characterized as endothelial cells based on their cobblestone morphology and surface expression of CD31, vWF and endothelial protein C receptor (EPCR) measured by flow cytometry (Fig. 4). See below for the protocol of flow cytometry.

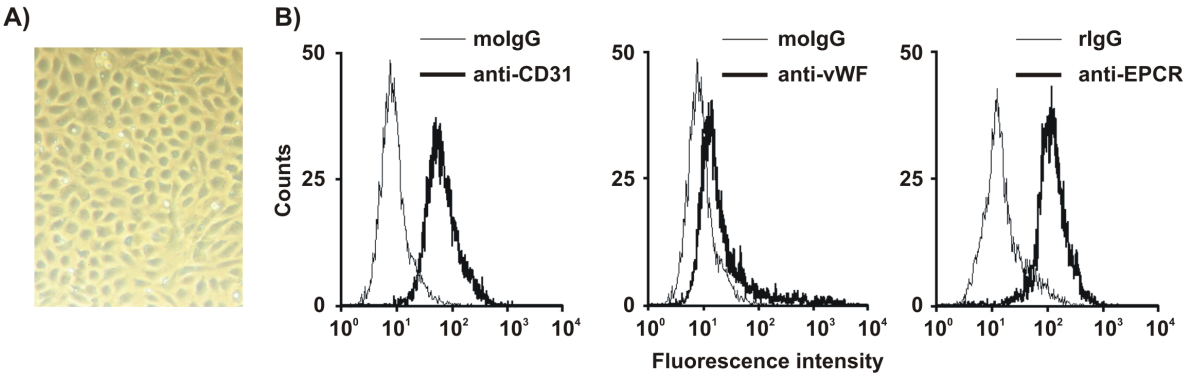

Figure 4. Endothelial cell characterization. A) Cells grow as a monolayer showing cobblestone morphology. Original magnification: 400x. **B)** Surface expression of CD31, vWF and EPCR was measured using monoclonal antibodies by flow cytometry. moIgG: mouse IgG, rIgG: rat IgG

2.2.2. Measurement of $[Ca^{2+}]_{ic}$

Expression of functional cysLT$_2$-R and PAR-1 was verified before each experiment by measuring the $[Ca^{2+}]_{ic}$ increase in response to receptor activation by 100 nM LTD$_4$ or 10 nM thrombin, respectively. HUVECs were cultured in an 8.5 cm^2 dish. Fluo4-AM (1 mM dissolved in dimethyl sulphoxid) was added to the medium at a final concentration of 2 µM. After homogenizing the dye by gentle shaking, the cells were incubated for 30 min at 37°C. Cells were rinsed twice with warm calcium buffer and kept in 1 ml buffer. Measurement was performed using a Zeiss Axiovert 200 microscope equipped with an LSM 510 laser scanner head and a heatable mounting frame. The stimulant was diluted in 1 ml warm buffer and added at the beginning of scanning. Picture series were recorded for 180 s. $[Ca^{2+}]_{ic}$ changes are expressed as mean fluorescence intensities of the scanned area over time. Representative signals are shown in Fig. 5.

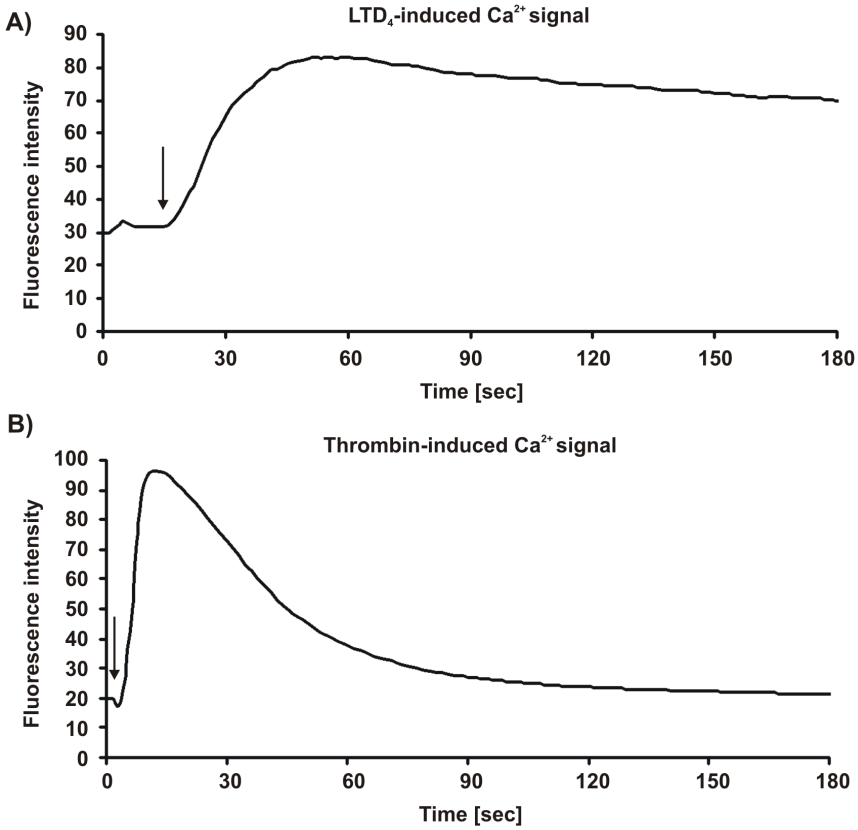

Figure 5. CysLT$_2$-R- and PAR-1-mediated Ca^{2+} signals. HUVECs were loaded with the Ca^{2+}-indicator Fluo4-AM and stimulated with **(A)** 100 nM LTD_4 or **(B)** 10 nM thrombin. The arrows show the time point of agonist addition. Changes in $[Ca^{2+}]_{ic}$ are in proportion to fluorescence intensity, which was recorded with a confocal laser scanning microscope every 1.5 s during a 3 min interval.

2.2.3. RNA extraction

For RNA extraction HUVECs were cultured in 8.5 cm^2 (or 21 cm^2) dishes. Medium was removed and the cells were washed twice with cold PBS. Remaining PBS was removed and cells were lysed in 350 µl (or 900 µl) TRIzol. Lysate was collected into an Eppendorf tube using a cell scraper, homogenised by vortexing and transferred into a Phase Lock Gel tube (Eppendorf, Hamburg, Germany) tube. 0.2 volume of chloroform was added to the lysate, and phases were mixed by vigorous shaking for 1 min. Phases were separated by centrifugation at 18,000 *g* for 15 min at 12°C. The upper aqueous phase containing the RNA (~200 µl) was mixed with 96-100% ethanol (110 µl) and transferred onto RNeasy MinElute Spin Columns of RNeasy MicroKit (8.5 cm^2 dish) or RNeasy MiniKit (21 cm^2 dish). Further steps were carried out according to the manufacturer's manual. RNA was eluted by centrifugation with 2x12 µl RNase-free water (1 min, 10,000 *g*, 20°C). RNA concentration was measured at 260 nm with the NanoDrop spectrophotometer and adjusted to 100 ng/µl for (q)RT-PCR or 625 ng/µl for microarray.

2.2.4. Microarray

To determine gene expression patterns of the agonists, microarray analyses were performed. For standardization, only male cords were used. HUVECs were cultured in 21 cm^2 dishes for 8 days in the 1st passage. After stimulation with 100 nM LTD_4, 10 nM thrombin or solvent control, RNA was extracted as described and RNA integrity was determined on RNA 6000 Nano LabChips using the Agilent BioAnalyzer 2100 machine (Agilent Technologies, Palo Alto, CA) according to the manufacturer's instructions.

RNA was reverse transcribed into cDNA, which was then *in vitro* transcribed into cRNA. After fragmentation, cRNA was hybridized on HG-U133A chips. Genechips were scanned with the GeneChip® Scanner 3000 (Affymetrix, Santa Clara, CA) using the GCOS software. cRNA preparation, hybridization and scanning were kindly performed by Christine Ströhl and Markus Hildner according to standard Affymetrix protocols and using the microarray platform of the Institute for Vascular Medicine.

2.2.4.1. Terms

Probe: a 25mer oligonucleotide synthesized on the array surface.
Perfect match probe (PM): a 25mer that is complementary to the sequence to be hybridized.
Mismatch probe (MM): a 25mer with a mismatch in the 13th position; the 13th nucleotide is the Watson-Crick complement of the 13th base in the perfect match probe. Using the mismatch probes makes it possible to estimate a cross-hybridization background for each PM sequence.
Probe cell: a square-shaped spot on the array containing probes with a unique sequence.
Probe pair: a PM and a MM probe in a probe set that have the same sequence except for the 13th nucleotide in the MM.
Probe set: 11 probe pairs (on the U133A array) corresponding to the same target sequence (transcript).

2.2.4.2. Raw data: signal, call, detection p value

The scanner generates the .dat file containing the fluorescence intensity of the chip in a range of 0-65,000. It is displayed as an image with 256 colors, which makes it possible to check visible quality of the chip (presence of bubbles or scratches, high regional or overall background) and to control grid alignment. In the grid each square delineates a probe cell (7x7 pixels). A background value is calculated for every cell (global background adjustment). Noise

value is calculated from the global background variation. After background subtraction and noise correction, the adjusted intensity is computed for each probe cell (stored in the .cel file). To adjust the PM intensity an ideal mismatch (IM) value is calculated: if PM > MM, IM = MM, if PM ≤ MM, an IM value is estimated using the one-step Tukey's biweight algorithm. The adjusted PM intensities are logarithmized, and a weighted mean is calculated for each probe set. The anti-log of this value is the signal intensity value. In order to make the arrays comparable, the mean signal intensity of every array was adjusted to a trimmed mean of 500. To this end, signal mean was calculated by discarding the highest and lowest 2% of all signal intensity values on a genechip, and the scaling factor (SF) for each array was computed: SF = 500 / trimmed signal mean. Every signal intensity value on a chip was multiplied by the SF and this value was given as the signal value for analysis.

For each probe pair, a relative difference of the PM and MM intensities can be calculated. Using the one-sided Wilcoxon's signed rank test a *p* value can be calculated for the whole probe set. It describes the significance of the result for the whole probe set. Setting two cut-off values defines three intervals for making the call. The call can answer the question of whether a certain transcript is detectable in the sample. With these three intervals the program defines three calls: Absent, if $p > 0.065$ (not detectable, i.e. not expressed or the expression is below the threshold level); Present, if $p \leq 0.05$ (the transcript is expressed and detectable); in uncertain cases (e.g. noisy probe sets), where $0.05 < p \leq 0.065$, a Marginally present call is given. The three parameters belonging to each probe set (signal value, detection *p* value and call) are stored in the .chp file and can be exported for analysis with other programs.

2.2.4.3. Genechip quality control

Quality control parameters, i.e. background, SF, noise, 3'/5' ratios of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and actin, spiked oligo controls, and % Present calls, were checked for each sample before the analysis. To ensure that the quality of the RNA samples and assays was acceptable and comparable, 3'/5' ratios of housekeeping genes, SF and % Present calls were compared from each experiment (Table 3).

2.2.4.4. Analysis

Raw data were filtered according to stringent filter criteria. To compare stimulated samples with control, the following criteria were set: signal intensity value >150 and detection *p* value <0.065 for at least three of four samples in either the control or the stimulated group; and fold change calculated from the groups' signal means >2.5. The signal intensity value of 150 was chosen based on the results of previous experiments, and the detection *p* value limit was chosen as recommended by Affymetrix. This combination gave a fine threshold estimation to exclude probe sets with Absent call and to keep data of genes with low expression. To declare a probe set regulated, the fold change threshold was arbitrarily set to 2.5. Filtered probe set lists were further analyzed using the GeneSpring(TM) expression analysis software (version 7.2, Silicon Genetics, Redwood City, CA). Raw data were normalized per probe set, and differentially expressed probe sets were identified using a one-way, two-level analysis of variance (ANOVA) with Benjamini-Hochberg correction for multiple testing ($p < 0.05$). In case of genes represented by more than one probe set, the one with the lowest ANOVA *p* value was used for heatmap generation. Lists of potentially regulated genes were generated by filtering data without further statistical analysis. Filtering criteria are given in table legends.

Sample	3'/5' ratio of GAPDH	3'/5' ratio of Actin	Scaling Factor (SF)	% Present Call
Affymetrix recommendation	<3	<3	SF discrepancies should be <3-fold	>30%; similar within one experiment
C1	0.81	1.02	2.041	49.2
L1	0.81	1.05	2.143	49.9
T1	0.90	1.03	3.026	44.6
C2	0.89	1.10	3.209	41.3
L2	0.91	1.13	2.751	49.4
T2	0.84	1.26	2.685	49.0
LT2	0.91	1.00	2.663	46.1
C3	0.83	1.08	1.674	51.7
L3	0.83	1.07	2.052	51.0
T3	0.87	1.15	1.779	50.3
LT3	0.81	1.09	1.997	49.5
C4	0.79	1.12	1.687	46.6
L4	0.76	1.08	1.372	50.3
T4	0.73	1.01	1.603	47.1
LT4	0.91	1.00	1.920	47.7

Table 3. Numerical quality control parameters of the genechips of the 1 h samples.
C: control, L: LTD_4, T: thrombin, LT: LTD_4 + thrombin

Probe sets were designated using the Affymetrix annotation tables. Analysis of data on LTD_4 plus thrombin treated samples compared to LTD_4 or thrombin alone was kindly performed by Dörte Radke.

2.2.5. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

In order to confirm the results obtained by microarray analysis and to quantify gene regulation, qRT-PCR analyses were performed. Total RNA was reverse transcribed, and cDNA transcript number was evaluated by quantitative PCR. Standard fragments for the calibration were prepared by semi-quantitative PCR.

2.2.5.1. Rerverse transcription

For 60 µl reaction, 3 µg RNA dissolved in 30 µl water was mixed with oligo $dT_{(12\text{-}18)}$ primer (8 µl 25 pmol/µl) and denatured (4 min, 72°C). After cooling down to 42°C, 22 µl reverse-transcription (RT) mix was added. RT ran for 1 h at 42°C and a final denaturation step was performed at 94°C for 10 min.

RT mix for 60 µl reaction:

1.15 µl nuclease-free water
11.70 µl 5x RT buffer
3.90 µl dNTP mix (2.5 mM of each dNTP)
1.45 µl RNase Inhibitor (RNaseOUT; 40 U/µl)
1.45 µl 0.1 M dithiotreitol
1.75 µl AMV (15 U/µl)
0.60 µl BSA (10 µg/µl)

2.2.5.2. Primer design

Primers were either taken from literature or selected using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The following aspects were considered: a primer length of 18–27 nucleotides; a product size of 100–300 bp; a T_m (melting temperature) for the primer of 67–71°C; a T_m for the product of <86°C; a GC content of the primer of 50–65%; both primers had G or C at the 3' end. In case of genes with more transcript variants, the primer pair was oriented to recognize all known splice variants. For establishing the PCR conditions, the annealing temperature (AT) was calculated as follows:

AT = (T_m-3' primer + T_m-5' primer) / 2–4, where primer T_m = 4(G+C) + 2(A+T)

Depending on the first PCR result, conditions were empirically optimized for each primer. Primers and conditions used in this work are summarized in Table 4.

2.2.5.3. Semi-quantitative PCR

For primer establishment and standard fragment preparation for qRT-PCR, semi-quantitative RT-PCR was performed with the following profile: initial denaturation 30 s 99°C, 2.5 min 94°C; cycling: 30 s 94°C, 30 s AT, 30 s 72°C and a final elongation: 3 min 72°C. The reaction ran for 25-35 cycles depending on the estimated expression of the gene. At the end of the reaction, samples were mixed with 10x stop solution. The size and quality of the amplified band(s) were analyzed by electrophoresis on 2w/v% agarose gel. For size orientation a DNA mass ladder was used. The bands were visualized by UV light and cut out from the gel. To elute the cDNA for reamplification, gel pieces were incubated in 200 µl nuclease-free water at 4°C overnight.

PCR mix for 25 µl reaction:

- 14.1 µl nuclease-free water
- 1.0 µl RT-cDNA
- 2.0 µl dNTP (2.5 mM of each dNTPs)
- 0.8 µl 50 mM $MgCl_2$ (final concentration: 1.5 mM)
- 4.0 µl Primer mix (3 µM of both primers)
- 2.5 µl 10x PCR buffer
- 0.3 µl BSA (10 µg/µl)
- 0.3 µl Platinum Taq polymerase (5 U/µl)

2.2.5.4. Reamplification and clean-up of the standard fragment

To gain a considerable cDNA amount, the fragment cut out from the gel was reamplified. The PCR profile was optimized according to the results of the establishing PCR. The size and quality of the amplified fragment was checked by electrophoresis on 2w/v% agarose gel using 2 µl from the sample. If the reamplification led to unspecific bands, the reaction was repeated with more stringent conditions by adjusting the AT or the $MgCl_2$ concentration. The fragment was cleaned up using the MSB Spin PCRapace purification kit according to the manufacturer's instruction. cDNA concentration was measured at 260 nm with the NanoDrop spectrophotometer and adjusted to 70 ng/µl.

PCR mix for 100 µl reaction:

56.0 µl from the eluted cDNA
18.5 µl primer mix (3 µM of both primers)
10.0 µl dNTP (2.5 mM of each dNTPs)
10.0 µl 10x PCR buffer
3.5 µl 50 mM $MgCl_2$
1.0 µl BSA (10 µg/µl)
1.0 µl Platinum Taq DNA Polymerase (5 U/µl)

2.2.5.5. Sequencing

The PCR product was sequenced by MWG-Biotech (Ebersberg, Germany). Samples and primers were sent to the firm according to its protocol. The provided sequence was verified using the blastn program (http://www.ncbi.nlm.nih.gov/BLAST/).

2.2.5.6. qPCR

qPCRs were carried out with a Rotor-Gene 2000 thermocycler (Corbett Research, Mortlake, Australia) and SYBR Green I as fluorescent dye. Reaction conditions were individually optimized for each primer pair. The amplified fragments were quantified via standard curves in the range of 8-2,500,000 ssDNA molecules in 40-cycle-reactions. Standards were diluted from the purified and sequenced fragments according to the following conversion: 1 µg 1,000 bp dsDNA = 1.52 pmol = 3.04 pmol ssDNA. Quality of the reactions and fragments was verified by analyzing amplification efficiency and melting curves. GAPDH was measured as reference gene for normalization. Results are expressed as target gene transcripts / 1,000 GAPDH transcripts.

qPCR mix for 25 µl reaction:

12.75 µl nuclease-free water
2.00 µl RT-cDNA
2.00 µl dNTP (2.5 mM of each dNTPs)
0.90 µl 50 mM $MgCl_2$
4.00 µl primer mix (3 µM of both primers)
2.50 µl 10x PCR buffer
0.25 µl BSA (10 µg/µl)
0.30 µl Platinum Taq DNA polymerase (5 U/µl)
0.30 µl SYBR Green I (diluted 1:400 in RT buffer)

Gene	Primer sequences	Product size [bp]	AT [°C]
ADAMTS1	5': CCATGTAGCCCAGATTCCACCT	224	64
	3': CGCTGTTTCACTTCGATGTTGG		
CD55	5': TGGCCCACCACCTGAATGC	290	66
	3': GCGTCCCAAGCAAACCTGTCAAC		
CXCL2	5': GAATCTACTTGCACACTCTCCC	156	63
	3': GACATGTGATATGTCATCACGAAG		
$CysLT_1$-R	5': GTGCCGCCTCAGCACCTATGC	545	68
	3': CGGACTTCTGCATTCTAAGGACAG		
$CysLT_2$-R	5': GATCTCCTGTTCATAAGCACGCTTC	385	69
	3': GCAGGCAGCCCACCACCAAGGC		
DSCR1 (pan)	5': GCACGCAGCGACTGACACCA	174	66
	3': CCTCGTCGCGTGCCAGTTCA		
1-7-8-9	5': GTGTTCGTGGACGGCCTGTG	548	67
	3': CCTCGTCGCGTGCCAGTTCA		
2-7-8-9	5': CAGACATTGAGCGGCGACGTG	618	67
	3': CCTCGTCGCGTGCCAGTTCA		
3-7-8-9	5': GGCTGCTGCCAATACAACACCAC	309	64
	3': TTCCATCCCACTGGCGGAGAG		
5-7-8-9	5': CGTGGGTCTGTAGCGCTTTCACTG	389	64
	3': TTCCATCCCACTGGCGGAGAG		
6-7-8-9	5': CCGCGCTTGCTAGCAGTGAAC	368	64
	3': TTCCATCCCACTGGCGGAGAG		
EGR1	5': GAGTTGGAATGTTGTAGTTACC	158	58
	3': GCAATAAAGCGCATTCAATGTG		
EGR2	5': ACTCTCCGCCACCGCCTCCT	281	63
	3': CACCCGCAGGGTGTCCAGTG		
EGR3	5': TCACCACCTGCGCCTGAGGA	192	68
	3': CTGGCCGGCGTGAAAGGTTG		
EGR4	5': GGCCTCCCTGGGCTCCTGAC	175	65
	3': CAGCCACGCCACTGCTTCCA		
E-selectin	5': GCTCTGCAGCTCGGACATGTGG	201	66
	3': GGCAGCTGCTGGCAGGAACA		
GAPDH	5': TCGGAGTCAACGGATTTGGTCGTA	520	66
	3': ATGGACTGTGGTCATGAGTCCTTC		
IL-8	5': CTTGGCAGCCTTCCTGATTT	265	59
	3': CTCAGCCCTCTTCAAAAACT		
NR4A1	5': CCTCCACGCCCAGCTTCCAG	172	69
	3': GGGCCGGTGGGAGGACTGAA		
NR4A2	5': GTCCGGACAGCAGTCCTCCA	172	65
	3': GCTGTGCTGCACCTGGAAGC		
NR4A3	5': CGAACCTGCGAGGGCTGCAAG	308	60
	3': GAGTCTGTTAAAGCTCGGACAAG		
TF	5': CGATCTCGCCGCCAACTGGT	214	66
	3': GATTGACGGGTTTGGGTTCCCACT		

Table 4. Primers used in PCRs. Primer sequences from the literature are: $cysLT_1$-R, $cysLT_2$-R, GAPDH [Spanbroek 2003], CXCL2 [Zhao 2004], NR4A3 5' [Martinez-Gonzalez 2003], NR4A3 3' [Satoh 2002].
AT: annealing temperature

2.2.6. Protein extraction and concentration measurement

2.2.6.1. Lysis in Laemmli buffer

HUVECs were grown in 8.5 cm^2 dishes. Medium was removed and cells were rinsed twice with ice-cold PBS. Cells were lysed in 150 µl Laemmli buffer. The lysate was collected with a cell scraper, transferred to a 1.5 ml tube, homogenized by thorough pipetting and boiled for 5 min at 100°C. To fragment genomic DNA, the lysate was sonicated 3x 5 s (G. Heinemann Ultraschall- und Labortechnik, Schwäbisch Gmünd, Germany) on ice. After centrifugation (10,000 *g*, 2 min, 20°C) the supernatant was aliquoted and stored at –20°C.

2.2.6.2. Lysis in Tris-Triton buffer

HUVECs were grown in 21 cm^2 dishes. Medium was removed and cells were rinsed twice with ice-cold Tris buffer. Cells were scraped in 1 ml Tris-Triton buffer, transferred to a 1.5 ml tube, and centrifuged (500 *g*, 5 min, 4°C). Supernatant was removed and cells were lysed in 70 µl Tris-Triton buffer on ice for 15 min by intermittent vortexing. Lysate was centrifuged at 15,000 *g* for 15 min at 1°C. 60 µl from the supernatant was mixed with 15 µl 5x Laemmli buffer and boiled for 5 min at 100°C. After centrifugation (10,000 *g*, 2 min, 20°C) it was aliquoted and stored at –20°C.

2.2.6.3. Protein concentration measurement

Protein concentration was determined by amidoblack staining. 0.1 mg/ml – 1.0 mg/ml BSA in Laemmli buffer was used as standard. Samples were measured both undiluted and diluted in 1:10. 1-µl drops were applied onto nitrocellulose membrane. After drying, the membrane was stained in amidoblack staining solution for 5 min, rinsed with water, and destained. The wet membrane was put between clear foils and scanned using a common flatbed scanner at 180 dpi, greyscale. Dots were densitometrically analyzed by the IP LabGel program (Lifescience Software Resource, Long Lake, USA), and concentration was calculated by interpolating onto a standard curve. Lysate concentration was adjusted to 1 µg/µl.

2.2.7. SDS-polyacrylamide gel electrophoresis and Western blot

For gel preparation, electrophoresis and transfer, the Mini-PROTEAN 3 System (Bio-Rad, Munich, Germany) was used. HUVEC extracts (10 µg protein in 10 µl) were separated on a

10% denaturing polyacrylamide gel at 120 V for 90 min, and transferred onto nitrocellulose membrane (GE Healthcare, Freiburg, Germany) by 300 mA for 90 min. For estimating the molecular weight, biotinylated protein ladder (Cell Signaling, Danvers, MA) was used.

2.2.7.1. Ponceau Red staining

After the transfer, protein bands were visualized to determine if they had migrated uniformly and evenly. The membrane was stained in Ponceau Red staining solution for 5 min at 20°C on a shaker and then destained with distilled water until the protein bands were clearly visible. The wet membrane was put between clear foils and scanned by a common flatbed scanner.

2.2.7.2. Immunostaining

The membrane was incubated in blocking solution at 4°C overnight. For early growth response 1 (EGR1), anti-EGR1 antibody was diluted in blocking solution (Table 5), and the membrane was incubated for 1 h at 20°C. For TF, anti-TF antibody was diluted in blocking solution (Table 5), and the membrane was incubated for 2 h at 20°C. After washing with Tween-PBS (3x 10 min, 20°C), the membrane was incubated with secondary antibody and with anti-biotin antibody for the protein ladder for 1 h at 20°C (Table 5). The membrane was washed with Tween-PBS (3x 10 min, 20°C) and finally with PBS (5 min, 20°C). Immunoreactive bands were detected by CDP-Star reagent (Applied Biosystems, Darmstadt, Germany). CDP-Star is a chemiluminescent substrate for alkaline phosphatase (AP). Upon dephosphorylation by AP the substrate changes to an unstable intermediate product whose decomposition results in light emission. Light production lasted up to 24 h allowing optimal detection using X-ray films (GE Healthcare). Films were developed using Kodak reagents.

Antibody		**Dilution**
rabbit anti-EGR1	Santa Cruz Biotechnology (Santa Cruz, CA)	1:6,000
rabbit anti-TF	American Diagnostica (Pfungstadt, Germany)	1:4,000
goat anti-rabbit AP	Santa Cruz Biotechnology	1:5,000
goat anti-biotin AP	Cell Signaling	1:5,000

Table 5. Antibodies and their dilution used in Western blotting. AP: alkaline phosphatase

2.2.8. IL-8 ELISA

Cells were cultured in 24-well-plates. Prior to stimulation, the medium was replaced with fresh medium without growth factors in order to remove the IL-8 produced continuously by the cells. HUVECs were stimulated with 100 nM LTD_4 or with 100 U/ml TNFα or with 0.05% ethanol as solvent control. After the indicated intervals, supernatants were collected and the cells were lysed for qRT-PCR. 1 ml of the supernatant was centrifuged at 500 *g* for 5 min at 4°C to remove cell debris, and aliquots were stored at –80°C until processing. For IL-8 protein measurement the BD OptEIA Human IL-8 ELISA Kit II was used, which contains IL-8 specific capture antibody coated on a 96-well plate. Standards (in 0–100 pg/ml range) and sample dilutions were added to the wells. After washing, streptavidin-horseradish peroxidase conjugate mixed with biotinylated anti-human IL-8 antibody was added. The ELISA was developed using 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution. The reaction was stopped with 1 M phosphoric acid, and absorbances were measured at 450 nm with a microplate reader.

2.2.9. TF procoagulant activity assay

HUVECs were cultured in 8.5 cm^2 dishes and lysed in 150 µl TF lysis buffer. Lysate was harvested with a cell scraper and sonicated (5x 3 s) on ice. Samples were spun down and stored at –80°C until processing. Procoagulant activity was determined by a chromogenic assay (Actichrome TF), which measures the peptidyl activity of human TF. Lysates were mixed with human FVIIa and human FX and incubated at 37°C, allowing the formation of the TF/FVIIa complex, and the complex to convert the human FX to FXa. The amount of FXa generated was measured by its ability to cleave a highly specific chromogenic substrate (Spectrozyme FXa) added to the reaction mixture. The cleaved substrate released a para-nitroaniline chromophore into the reaction solution, thus changing its color to yellow. The reaction was stopped by adding glacial acetic acid, and absorbance was read at 405 nm by a microplate reader. TF concentration was calculated from a standard curve generated using known amounts of active human TF.

2.2.10. Flow cytometry

To examine the surface expression of marker molecules for EC characterization, cells were detached by incubation in PBS containing 0.2% EDTA for 5 min at 37°C. For measurement of CD55 expression, HUVECs were stimulated with 100 nM LTD_4 or 10 nM thrombin for various time points, and cells were harvested by trypsin-EDTA. 2x 0^5 cells / samples were stained with specific monoclonal antibodies or appropriate isotype controls (Table 6) for 15 min on ice. After washing with FACS buffer (400 *g*, 5 min, 4°C), cells were incubated with fluorochrome-conjugated secondary antibodies (Table 6) for 15 min on ice. Cells were washed, resuspended in 500 µl FACS buffer and measured with a FACSCalibur flow cytometer (BD Biosciences). Cell population was defined based on forward scatter – side scatter characteristics, and 10,000 cells were routinely counted. Data were analyzed with the CellQuest program.

Antibody		Dilution/concentration
rat anti-EPCR	Sanbio (Beutelsbach, Germany)	5 µg/ml
rat IgG	R&D Systems	5 µg/ml
goat anti-rat FITC	Dianova	1:50
mouse anti-CD31 (IgG1)	DakoCytomation (Hamburg, Germany)	1:100
mouse anti-vWF (IgG1)	DakoCytomation	1:100
mouse IgG1	DakoCytomation	1:100
goat anti-mouse PE	Dianova	1:100
mouse anti-CD55 (IgG2a)	BD Biosciences	5 µg/ml
mouse IgG2a	BD Biosciences	5 µg/ml
rat anti-mouse IgG2a+b	BD Biosciences	1:20

Table 6. Antibodies used in flow cytometry. FITC: fluorescein isothiocyanate, PE: phycoerythrin

2.2.11. Immunofluorescence

Cells were cultured on plastic coverslips (Nunc, Wiesbaden, Germany) in 24-well-plates. After stimulation, the cells were rinsed with HBSS at 20°C and air-dried. Samples were stored at –80°C until processing. After thawing, the cells were allowed to rehydrate in PBS for 5 min at 20°C, fixed in 4% formaldehyde in PBS for 10 min at 20°C, and permeabilized in 0.5% Triton X-100 in PBS for 5 min at 20°C. The samples were incubated with anti-EGR1 (1:100 in 0.25% BSA-PBS) for 30 min at 20°C. After washing with PBS, the cells were incubated with Cy3-conjugated anti-rabbit antibody (1:600; Dianova, Hamburg, Germany) for 30 min at 20°C, and

the DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). The samples were scanned using a Zeiss Axiovert 200 microscope equipped with a LSM 510 laser scanner head.

2.2.12. Statistics

Data are expressed as mean ± SEM. Statistical evaluation was performed by Student *t* test for paired data with Microsoft Excel and a p value < 0.05 was considered significant.

Results

3.1. LTD_4-induced gene signatures

To identify $cysLT_2$-R-regulated genes in ECs, microarray experiments were performed. Changes in the expression of more than 14,500 genes could be monitored in parallel using the Affymetrix U133A genechips. HUVECs were stimulated with 100 nM LTD_4 or with 0.05% ethanol as solvent control. 10 nM (1 U/ml) thrombin was used as positive control. After stimulation, total RNA was isolated, reverse transcribed into cDNA and *in vitro* transcribed into cRNA, which was hybridized to the arrays. The samples were scanned and data were analyzed using the GeneSpring expression analysis software. Data are published in the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO) database under the accession number GSE3589.

The first experiment investigating the time points 1 h, 6 h and 24 h showed that the strongest changes in gene expression in response to LTD_4 occur at 1 h (Fig. 6). Therefore, three further experiments were performed at the 1 h time point, also including samples stimulated with 100 nM LTD_4 and 10 nM thrombin together to examine the possibility of cooperation between the agonists.

To identify LTD_4-regulated genes, four data sets from independent umbilical cord preparations were analyzed at the 1 h time point using stringent filter criteria. Pre-filtering threshold values were set as follows: signal value >150 and detection *p* value <0.065 at least in three of four samples in either the control or the stimulated group. Probe sets were further filtered by the fold change parameter calculated from the groups' signal means >2.5. The resulting list of probe sets was statistically tested using one-way ANOVA with correction for multiple testing (Benjamini-Hochberg). Differentially expressed genes were determined by the ANOVA *p* value ($p < 0.05$). LTD_4 significantly up-regulated 37 genes represented by 48 probe sets at least 2.5-fold, and there were no genes down-regulated >2.5-fold. LTD_4-induced genes encode, among others, transcription factors, e.g. members of the EGR (early growth response), NR4A (nuclear receptor subfamily 4 group A) and KLF (Krüppel-like factor) transcription factor families; chemokines, e.g. IL-8 and CXCL2 (CXC chemokine ligand 2); signalling molecules,

e.g. regulator of G protein signalling 2 and DSCR1 (Down syndrome critical region gene 1); the adhesion molecule E-Selectin; the metalloprotease ADAMTS1 (a disintegrin-like and metalloprotease with thrombospondin motif 1, type 1); growth factors, e.g. HBEGF (heparin-binding EGF-like growth factor) and bone morphogenic protein 2; and (TF). Several of these genes are implicated in inflammatory processes [Kachigian 2006, Martínez-Gonzalez 2005, Suzuki 2005, Gerszten 1999, Levi 2006] (Fig. 7). The expression patterns of these 37 up-regulated genes are displayed in Fig. 7, and Table 7 in the Appendix. For comparison, thrombin data for these genes are also shown. The heatmap shows normalized signal intensities encoded by colours: green represents no or very low expression, yellow shows medium expression and red corresponds to high expression.

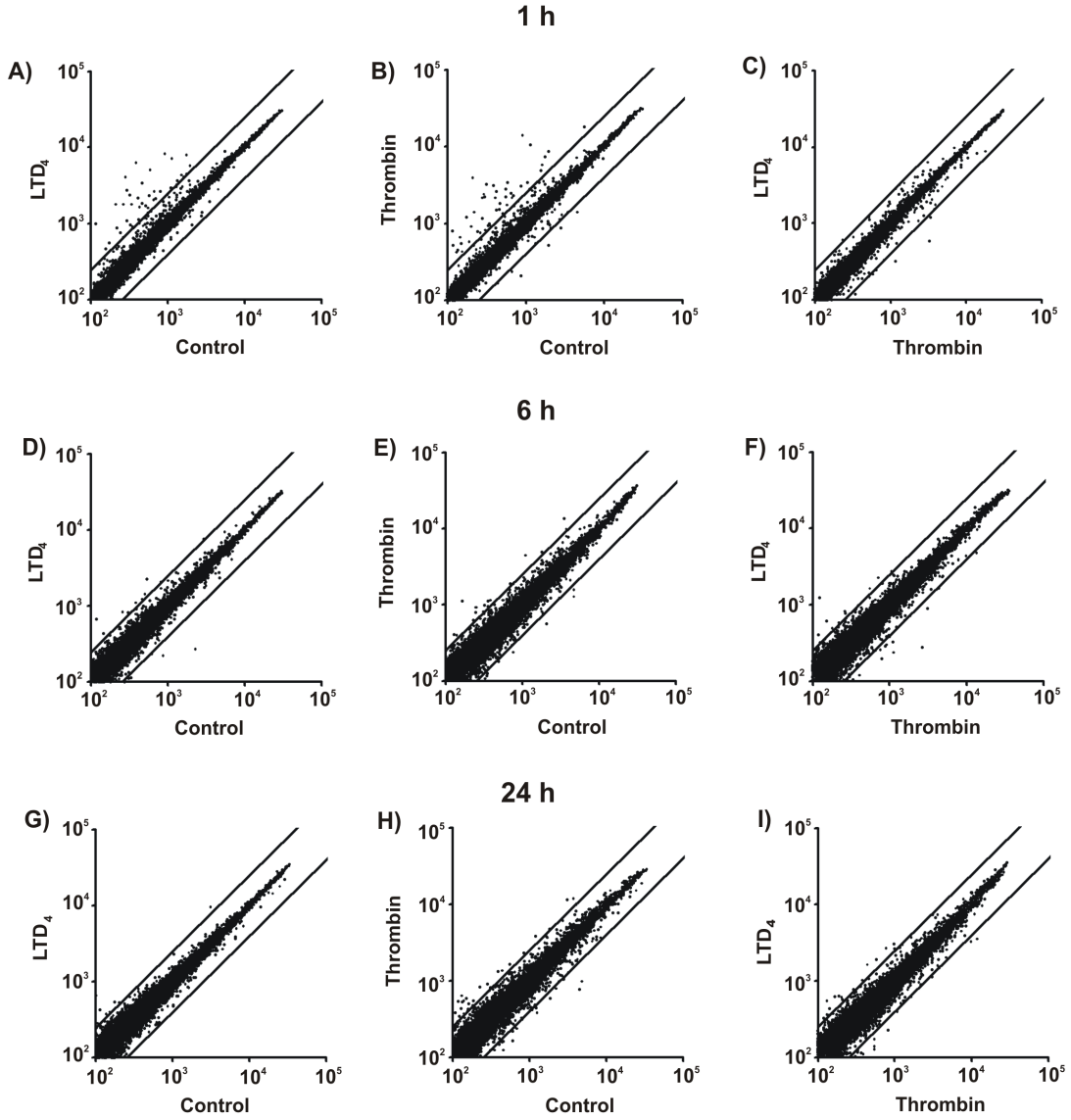

Figure 6. Gene expression patterns induced by LTD_4 and thrombin. HUVECs were stimulated with 100 nM LTD_4 or 10 nM thrombin and microarray analyses at the indicated time points were performed. Changes in gene expression are shown as scatterplots. Dots in plots A-C indicate means of signal intensities of four umbilical cords; in plots D-I signal values of single chip experiments are presented. Lines depict 2.5-fold changes.

3.1.1. LTD_4-induced early genes resemble those activated by thrombin

Early gene signatures in response to LTD_4 and thrombin were highly similar. Compared to the control, in both stimulated samples several genes were up-regulated (Fig. 6A and B, dots over the line depicting 2.5-fold changes). For thrombin, some genes were down-regulated at 1 h (Fig. 6B, dots beneath the line). If the LTD_4- and thrombin-induced gene expression patterns are plotted against each other, there are only few genes outside of the lines marking the 2.5-fold changes (Fig. 6C). Thus, under the given conditions the majority of genes regulated by the two agonists are identical, though the extent of induction is different.

For the analysis of thrombin-induced gene signatures the same criteria were used as for LTD_4. Thrombin treatment for 1 h resulted in 49 up-regulated and 7 down-regulated probe sets, corresponding to 34 up-regulated and 5 down-regulated genes. Genes up-regulated by LTD_4 were also up-regulated by thrombin and vice versa, even though the fold change in some cases did not reach our 2.5-fold threshold. For the 37 LTD_4-regulated genes displayed in Fig. 7, induction by LTD_4 and thrombin highly correlated (Pearson correlation coefficient, $r = 0.90$). When comparing the lists of genes regulated by LTD_4 and thrombin using less stringent criteria (fold change >2.0; without statistical test), the similarity between the effects of the two agonists becomes even more apparent (Tables 8 and 9 in the Appendix).

At 6 h and 24 h thrombin was a more potent agonist than LTD_4, but these late effects were weak compared to the 1 h results. Genes stimulated by thrombin at later time points were also induced by LTD_4, though at a lower level (Fig. 6D-I, and Tables 10-13 in the Appendix). However, since there were only few genes up-regulated by LTD_4 at 6 h and 24 h, these late gene signatures were not further analyzed.

3.1.2. LTD_4 and thrombin added together augment gene expression

On HUVECs both LTD_4 and thrombin activate G protein-coupled receptors, $cysLT_2$-R and PAR-1, respectively [Lötzer 2003, Coughlin 2000]. Stimulation of HUVECs with LTD_4 and thrombin alone resulted in a highly similar gene expression pattern. To examine whether concomitant activation of $cysLT_2$-R and PAR-1 leads to stronger gene activation, gene signatures of LTD_4 plus thrombin-stimulated cells were examined by microarray analysis (Table 14 in the Appendix).

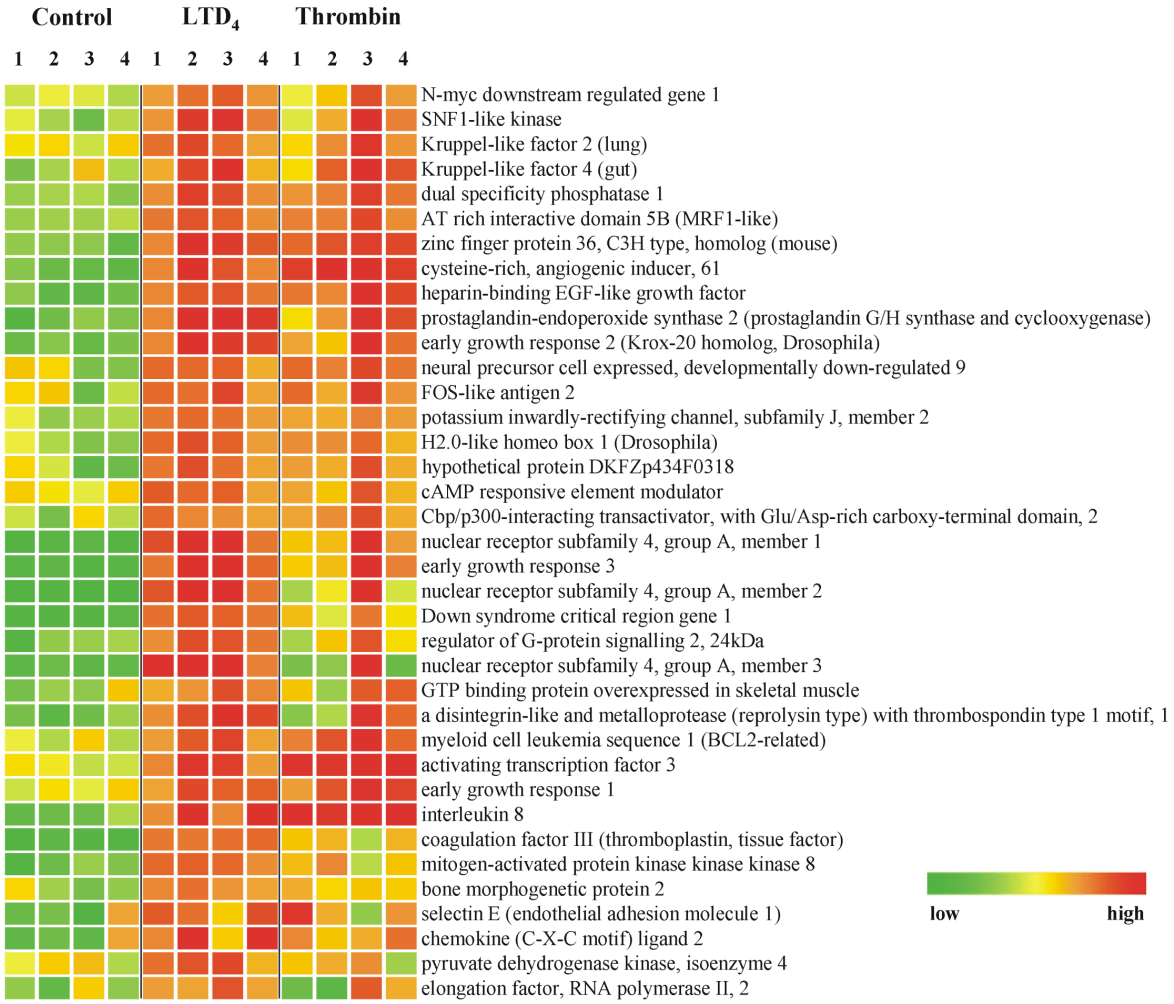

Figure 7. LTD_4-induced early genes in HUVECs. The heatmap shows normalized signal intensities of genes up-regulated >2.5-fold by 100 nM LTD_4 at 1 h. Data of these genes after stimulation with 10 nM thrombin are shown for comparison. Column numbers correspond to umbilical cord preparations. Signal intensity values are presented in Table 7 in the Appendix.

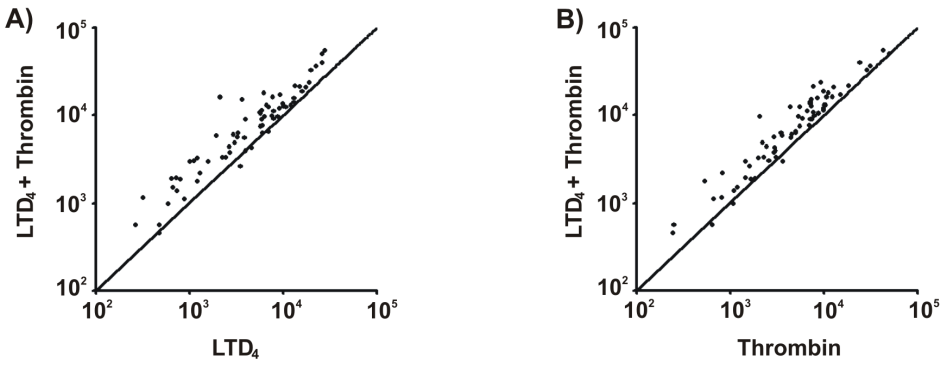

Figure 8. Enhancement of gene expression by LTD_4 + thrombin. The scatterplots show expression of the 68 probe sets induced more strongly via LTD_4 + thrombin together compared to LTD_4 (A) or thrombin (B) alone. Dots represent means of signal intensities of three umbilical cord preparations; lines indicate no change in expression.

Probe sets up-regulated by LTD_4 or thrombin alone were evaluated regarding their induction by the two agonists together. Probe sets up-regulated by at least one agonist were defined based on the previously described lists of significantly up-regulated probe sets by LTD_4 (48 probe sets) and by thrombin (49 probe sets). The combined list contained 68 probe sets encoding 50 genes. For these probe sets, induction by LTD_4 plus thrombin was significantly higher compared to induction by each agonist alone (Wilcoxon signed rank test, $p < 0.001$). Signal intensity pairs (both agonists together against a single agonist) of the 68 probe sets are displayed in Fig. 8 and Table 15 in the Appendix. Dots above the line represent probe sets with higher signal intensity value after LTD_4 plus thrombin treatment than in response to LTD_4 (Fig. 8A) or thrombin alone (Fig. 8B). These data show that LTD_4 and thrombin added together had a stronger effect on gene expression than the agonists alone and that this combination did not induce additional genes.

3.1.3. Validation of the microarray results by qRT-PCR

In order to confirm the microarray data and to further characterize the effects of LTD_4, the following genes were selected: NR4A1, NR4A2, NR4A3, EGR1, EGR2, EGR3, IL-8, CXCL2, DSCR1, ADAMTS1, E-Selectin, cyclooxygenase 2 (data not shown, K. Lötzer and A. J. R. Habenicht, manuscript in preparation) and TF. Induction of these genes was determined by qRT-PCR from RNA aliquots of the samples used for the microarray analyses. For qRT-PCR, external standards were prepared. Sequences of the standard fragments were confirmed by sequencing and blasting NCBI's Nucleotide Database, and via melting curve analysis. GAPDH was chosen as a reference gene for normalization. Relative expression is given as transcript numbers / 1,000 GAPDH transcripts for each gene (Fig. 9). Both microarray and qRT-PCR showed the same qualitative result. The LTD_4-triggered up-regulation of each gene was confirmed in four independent HUVEC cultures by the two methods, and in the further experiments. However, the extent of gene induction varied between the cord preparations (Fig. 9), which may be due to the biological variance. Kinetics of mRNA up-regulation for NR4A1, NR4A2, NR4A3, EGR1, EGR2, EGR3, IL-8, CXCL2, DSCR1, ADAMTS1, E-Selectin and TF was measured in two independent cord preparations (representative data are shown in Figures 12, 13, 14, 15, and 17).

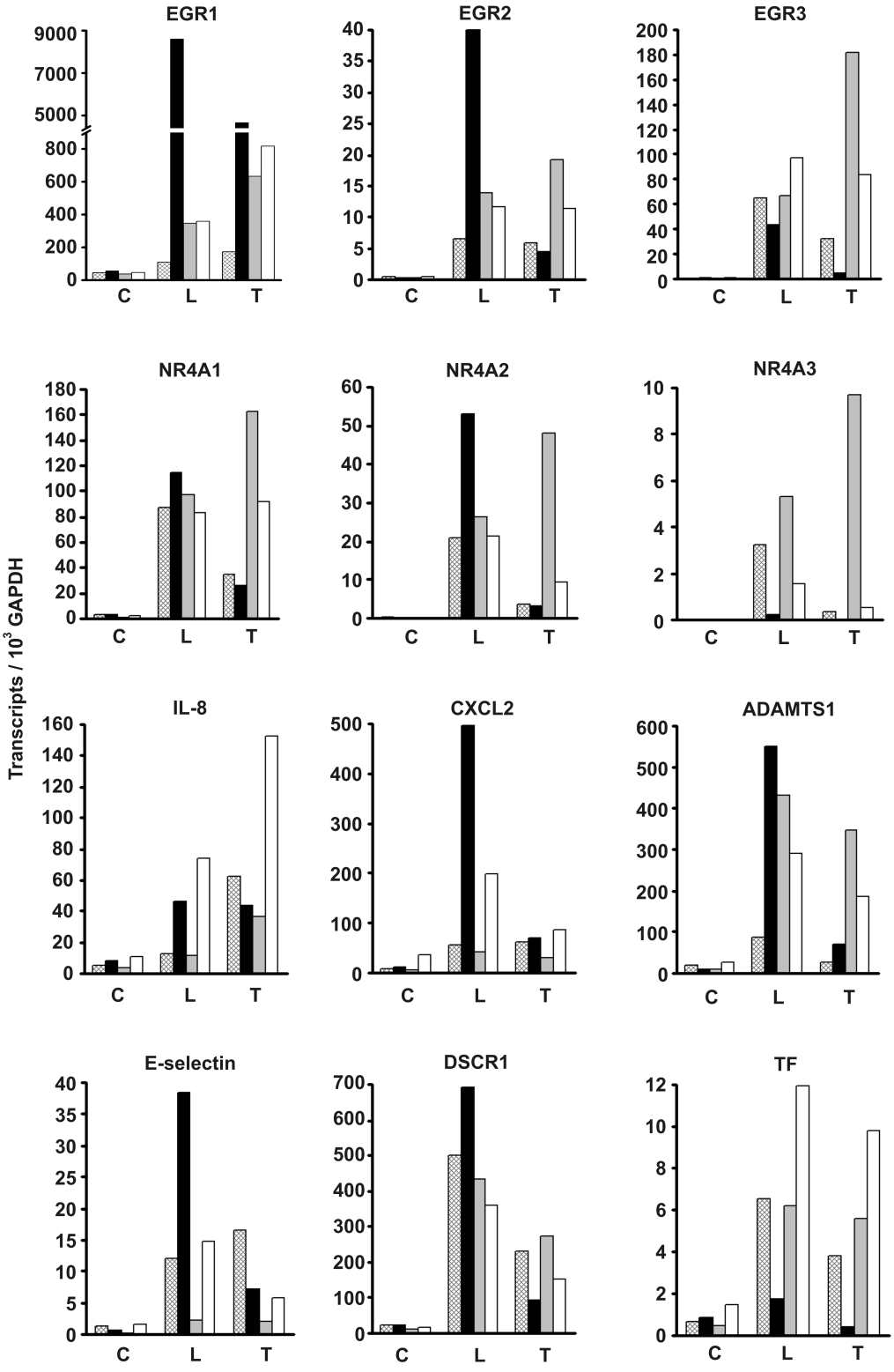

Figure 9. Validation of the microarray data via qRT-PCR. HUVECs were stimulated with 100 nM LTD_4 or 10 nM thrombin for 1 h. Aliquots of RNA samples prepared for microarray analyses were reverse transcribed into cDNA, and transcript levels of 12 genes were determined by qRT-PCR and normalized to GAPDH transcripts. Column patterns represent the four different umbilical cord preparations. C: control, L: LTD_4, T: thrombin. LTD_4-stimulated samples show in each case more transcripts compared to the corresponding control samples.

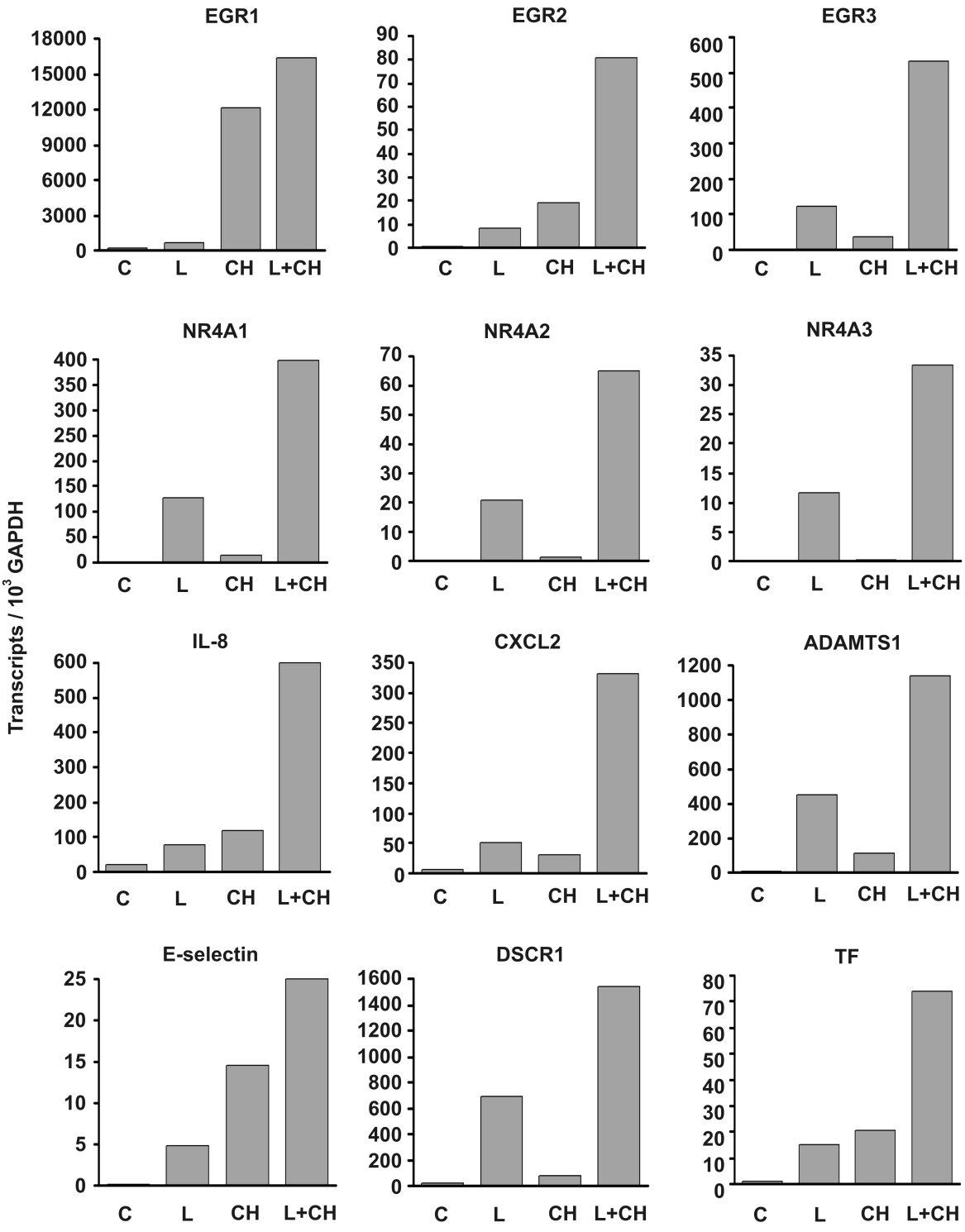

Figure 10. Effects of cycloheximide on LTD_4-induced genes. To identify immediate-early genes in response to LTD_4, cells were stimulated with 100 nM LTD_4 in the presence and absence of 10 µg/ml cycloheximide for 1 h and mRNA levels were measured by qRT-PCR. C: control, L: LTD_4, CH: cycloheximide, L+CH: LTD_4 + cycloheximide. Results of a single experiments are shown.

3.1.4. LTD_4 stimulates immediate-early genes

Transcript kinetics after LTD_4 stimulation was measured for each selected gene (see below). In all cases, transcript level peaked at approximately 60 min. Since many of the up-regulated genes are immediate-early genes in other agonist-response systems [Bravo 1990, Liu 2003], we examined whether the protein synthesis inhibitor cycloheximide blocks their induction by LTD_4. All 12 genes were induced by LTD_4, also in the presence of cycloheximide. Thus, transcription of these genes did not require newly synthesized proteins, and they are immediate-early genes in response to LTD_4 (Fig. 10).

3.1.5. LTD_4-induced gene signatures are the result of $cysLT_2$-R stimulation

In order to clarify receptor involvement in LTD_4-triggered gene signatures, cysLT-R transcript levels were measured in several umbilical cord preparations by qRT-PCR. Considerable amount of $cysLT_2$-R mRNA was detected in these cells, whereas $cysLT_1$-R was barely expressed (Table 16). Expression of the recently described putative cysLT-R, GPR17, was not determined by qRT-PCR. Both probe sets referring to GPR17 had Absent calls in our microarray experiments (data published in the NCBI GEO database, Acc. Nr. GSE3589), which, in concert with the receptor characterics obtained from transfectants [Ciana 2006], do not support a role for GPR17 in HUVECs. To gain further evidence for the selective activation

	Transcripts / 10^3 GAPDH (Mean ± SEM)
$CysLT_1$-R	0.0075 ± 0.0035
$CysLT_2$-R	9.03 ± 2.00

Table 16. CysLT-R transcripts in HUVECs. $CysLT_1$-R and $cysLT_2$-R transcripts were measured by qRT-PCR in HUVECs derived from four umbilical cord preparations used for the microarray experiments.

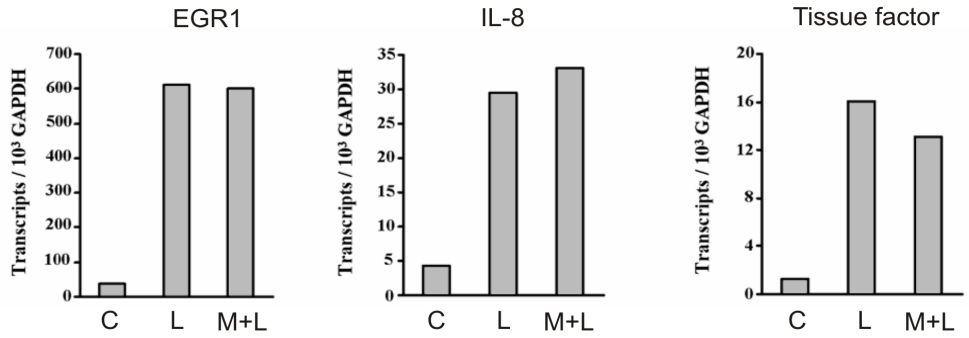

Figure 11. Effect of Montelukast on LTD_4-induced gene expression. HUVECs were preincubated with 2 µM Montelukast for 1 h, and after stimulation with 100 nM LTD_4 for 1 h, mRNA levels were measured by qRT-PCR. C: control, L: LTD_4, M+L: Montelukast + LTD_4. There was no difference between vehicle- and Montelukast-treated samples. Data are representative of three independent experiments.

of the cysLT$_2$-R, the effects of a cysLT$_1$-R inhibitor (which also inhibits GPR17) were examined on LTD$_4$-mediated transcriptional events. Similar to the Ca^{2+} signals [Lötzer 2003], LTD$_4$-initiated gene expression in HUVECs was not affected by Montelukast, as shown by qRT-PCR data of selected LTD$_4$-induced genes (Fig. 11). These data confirm that cysLTs act via the cysLT$_2$-R in HUVECs.

3.2. Characterization of LTD$_4$-induced early genes

3.2.1. LTD$_4$ induces the EGR and NR4A transcription factors

Among the 37 LTD$_4$-stimulated genes, 14 encode transcription factors (Fig. 7, Table 7 in the Appendix). Two transcription factor families, EGR and NR4A, were chosen for detailed analyses because they were strongly up-regulated as judged by the fold changes, and they may contribute to vascular inflammation [Khachigian 2006, Martínez-Gonzalez 2005].

Three members of the EGR family, EGR1, EGR2 and EGR3, were induced by LTD$_4$ as well as by thrombin (Fig. 7). The fourth member of this transcription factor family, EGR4, was also studied, however neither LTD$_4$ nor thrombin had an effect on its expression (data not shown). EGR1 was the most strongly up-regulated member, and it was also highly expressed in unstimulated cells (Fig. 12A, left axis). EGR1 showed maximal expression around 30 min and reached baseline level around 2 h. EGR3 was weakly expressed and EGR2 transcripts were barely detectable in control samples (Fig. 12A, right axis). EGR2 and EGR3 were induced with similarly rapid kinetics as EGR1, but at a lower level. Transcript levels of EGR1 and EGR3 differed by a factor of 10, and that of EGR1 and EGR2 by a factor of 100.

Since EGR1 was strongly induced at a high mRNA level by LTD$_4$, expression of EGR1 was also followed at the protein level by Western blotting (Fig. 12B). EGR1 protein was strongly up-regulated compared to the unstimulated control at 1 h. At later time points it appeared as a protein doublet on the blot and reached baseline level after 4 h. Cellular localization of EGR1 was studied by confocal laser scanning microscopy (Fig. 12C). In unstimulated cells, EGR1 was weakly expressed and was distributed throughout the cytoplasm, whereas in LTD$_4$- or TNFα-treated cells EGR1 showed stronger expression and was localized in the nucleus. Comparable induction of EGR1 by LTD$_4$ and TNFα was observed at 1 h both by Western blotting and microscopy.

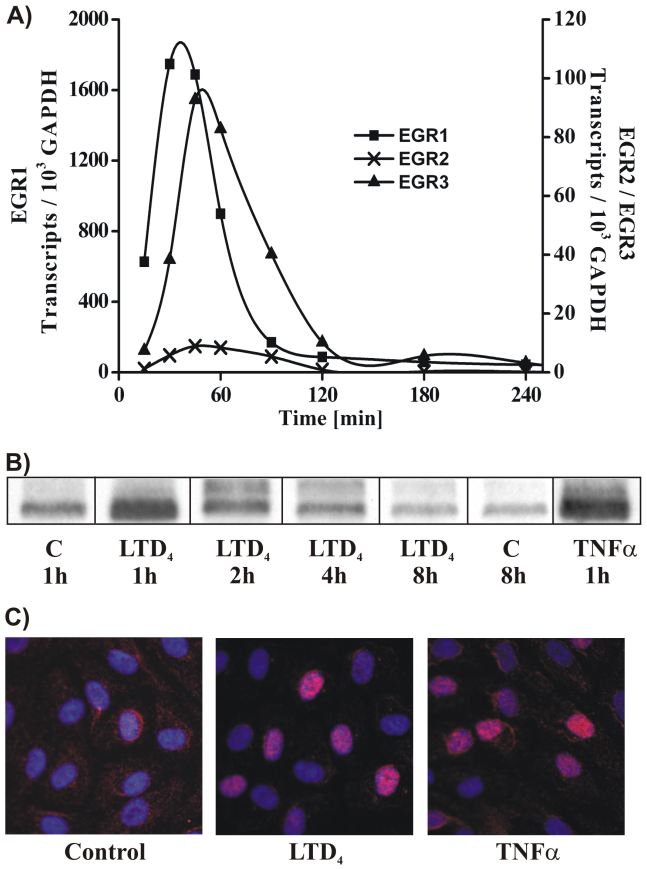

Figure 12. LTD_4 induces the EGR transcription factor family. A) HUVECs were stimulated with 100 nM LTD_4 and transcript kinetics of EGR1, EGR2 and EGR3 was measured by qRT-PCR. EGR1 was expressed and induced at an approximately 10-fold higher level than EGR3 and 100-fold higher level than EGR2. **B)** Protein kinetics of EGR1 in response to LTD_4 was determined by Western blotting. 100 U/ml TNFα was used as positive control. Results are representative of three independent experiments. **C)** Cellular localization of EGR1 protein was investigated by confocal laser scanning microscopy. After stimulation with 100 nM LTD_4 or 100 U/ml TNFα for 1 h, EGR1 localized in the nucleus, whereas in unstimulated samples its distribution was restricted to the cytoplasm. EGR1 is shown in red; DNA was stained with DAPI (blue).

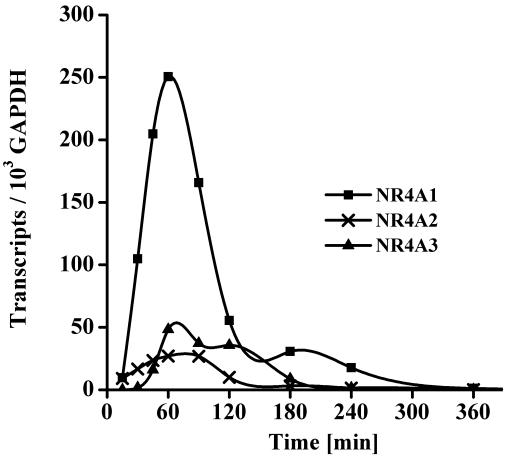

Figure 13. Induction of the NR4A transcription factors by LTD_4. HUVECs were stimulated with 100 nM LTD_4 and transcript kinetics of NR4A1, NR4A2 and NR4A3 was measured by qRT-PCR.

Within the NR4A transcription factor family all three members were induced by LTD_4 (Fig. 13). NR4A1 was the most strongly stimulated member, though differences in the extent of up-regulation were not as pronounced as in the EGR family. In stimulated samples, NR4A1 was expressed at ~5-fold higher level than NR4A2 and NR4A3. However, induction in the different umbilical cord preparations was variable. Though NR4A1 was strongly up-regulated by LTD_4 and the mRNA level in stimulated cells hinted at considerable protein amount, it was not detectable by Western blotting in our hands (data not shown).

Time point	Gene symbol	Transcription factor family	Motif and position
1 h	ADAMTS1	NR4A, EGR	AAA GGT CA found at –2721 GCG GGG GCG found at –1472
	ARID5B	NR4A	AAA GGT CA found at –4433
	DSCR1	NR4A	AAA GGT CA found at –4004
	EGR2	EGR	GCG GGG GCG found at –984
	F3	EGR	GCG GGG GCG found at –83
	GEM	EGR	GCG GGG GCG found at –70
	KCNJ2	NR4A	AAA GGT CA found at –4353
	KLF2	EGR	GCG GGG GCG found at –261
	NDRG1	EGR	GCG GGG GCG found at –50
	NEDD9	EGR	GCG GGG GCG found at –227
	NR4A1	NR4A	AAA GGT CA found at –2977
	NR4A2	EGR	GCG GGG GCG found at –1434
6 h	ALDH1A2	NR4A	AAA GGT CA found at –2032
	DSCR1	NR4A	AAA GGT CA found at –4004
	ZNF451	NR4A	AAA GGT CA found at –624
24 h	ANK2	NR4A	AAA GGT CA found at –2204

Table 17. EGR and NR4A binding sites in the promoter of LTD_4-induced genes. Promoter regions (defined as –5000 - +100 bp) of genes up-regulated >2.5-fold by LTD_4 at different time points were searched for binding motifs of the EGR and NR4A transcription factor families. Position of GCG G/TGG GCG (EGR) and AAA GGT CA (NR4A) sites is indicated. Gene symbols are: ARID5B: AT rich interactive domain 5B; F3: tissue factor; GEM: GTP binding protein overexpressed in skeletal muscle; KCNJ2: potassium inwardly-rectifying channel, subfamily J, member 2; NDRG1: N-myc downstream regulated gene 1; NEDD9: neural precursor cell expressed, developmentally downregulated 9; ALDH1A2: aldehyde dehydrogenase 1 family, member A2; ZNF451: zinc finger protein 451; ANK2: ankyrin 2, neuronal

Both transcription factor families have characteristic consensus DNA-binding sites: GCG G/TGG GCG (EGR family) [Silverman 1999] and AAA GGT CA (NR4A family) [Maira 1999]. In order to identify genes that might be regulated by these transcription factors in our system, genes up-regulated >2.5-fold by LTD_4 at 1 h, 6 h and 24 h were screened for these consensus sites. The promoter region was arbitrarily defined as –5000 - +100 bases. Search results are presented in Table 17. EGR binding sites were found in the promoter region of NR4A2, EGR2, KLF2, TF, whereas NR4A responsive element were found to be present in the NR4A1 and DSCR1 promoters. The ADAMTS1 promoter contains binding sites for both transcription factor families. It was not further investigated whether these binding sites are active in ECs.

3.2.2. LTD_4 up-regulates inflammatory genes in HUVECs

Among the LTD_4-induced genes there are several ones, which encode proteins that play important roles in inflammation of the vessel wall by attracting leukocytes and promoting their attachment of or by contributing to rearrangement of the extracellular matrix. IL-8, CXCL2, E-Selectin and ADAMTS1 were chosen for detailed analysis.

LTD_4 up-regulated IL-8 and CXCL2 (also called macrophage inflammatory protein 2α, Mip-2α), two chemokines of the same family. Both IL-8 and CXCL2 were also expressed in unstimulated cells, showed rapid induction in response to LTD_4 and returned to baseline expression at 4 h (Fig. 14). IL-8 protein was measured in HUVEC culture supernatants by ELISA. The fact that secreted IL-8 protein appeared to accumulate in the culture medium, whereas IL-8 transcripts in the cells did not, explains why the ratio of IL-8 protein amounts in stimulated versus unstimulated samples declined more slowly than the mRNA ratio (Fig. 14B). The maximal induction of IL-8 protein was detected 2 h after stimulation (Fig. 14C).

The adhesion molecule E-Selectin and the metalloprotease ADAMTS1 showed prolonged mRNA kinetics in response to LTD_4 when compared to other genes (Fig. 15). Both mRNAs reached maximum level between 60-90 min and returned to baseline level ~8 h after stimulation.

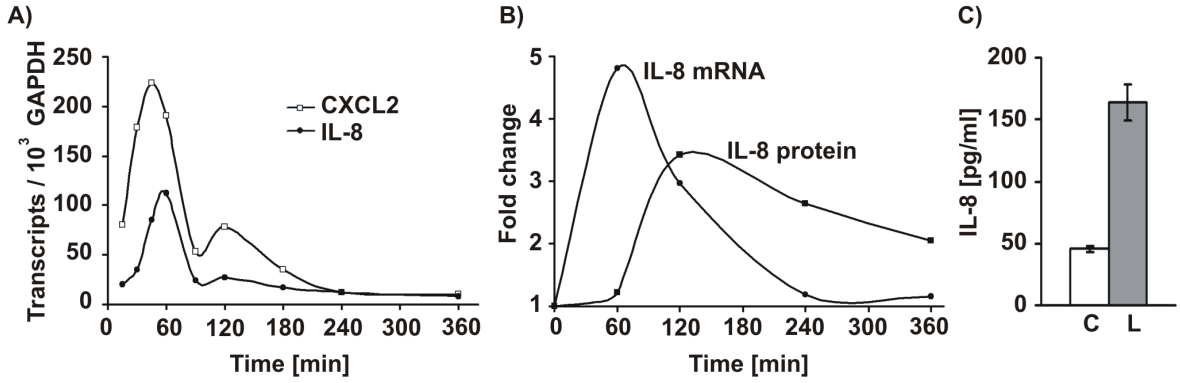

Figure 14. Up-regulation of IL-8 and CXCL2 by LTD_4. A) Induction of IL-8 and CXCL2 mRNA by 100 nM LTD_4 as measured by qRT-PCR. Both chemokines showed similar kinetics, but expression of CXCL2 was higher. **B)** Kinetics of fold changes in IL-8 mRNA and protein. Cells were stimulated with LTD_4 for the indicated time points, IL-8 protein was determined from culture supernatant by ELISA and cells were lysed for qRT-PCR analyses. **C)** IL-8 protein content of HUVECs supernatant measured 2 h after stimulation by LTD_4. Columns show means of quadruplicate samples ± SEM ($p<0.005$; Student t test). Data are representative of three independent experiments.

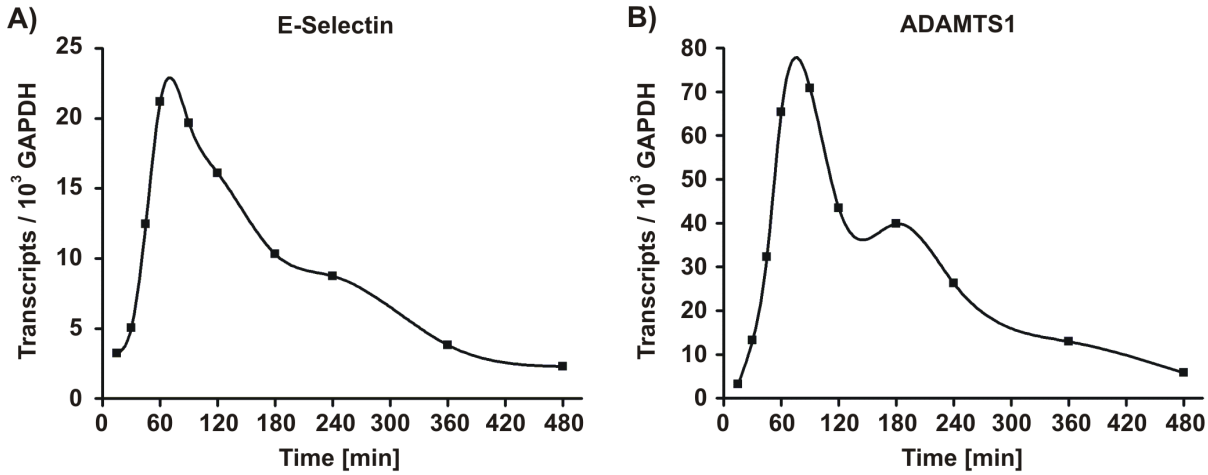

Figure 15. E-Selectin and ADAMTS1 kinetics upon LTD_4-stimulation. HUVECs were stimulated with 100 nM LTD_4 and transcript levels of E-Selectin (A) and ADAMTS1 (B) were determined by qRT-PCR.

3.2.3. LTD_4-mediated induction of DSCR1

DSCR1 encodes a protein — also called calcipressin — that is an endogenous inhibitor of calcineurin [Parry 2003, Harris 2005]. Calcineurin is a phosphatase and acts as an essential regulator of the transcription factor NFAT (nuclear factor of activated T cells). Upon dephosphorylation, NFAT translocates to the nucleus and regulates transcription of inflammatory genes [Hogan 2003]. DSCR1 was strongly induced by LTD_4 in HUVECs (Figures 7 and 9), which raises the possibility that DSCR1 is part of an endogenous feedback mechanism of inflammation.

Due to alternative splicing, DSCR1 has several transcript variants (TVs) with tissue or cell type-specific distribution [Harris 2005]. The DSCR1 gene encodes nine exons according to the NCBI RefSeq Database, though publications generally mention only seven. Exon 4 has not yet been detected at the protein level and exon 6 has been cloned only recently (Fig. 16A). Each TV consists of four exons. The first exon in the TVs is variable, but the last three, exons 7–9, are constant. This common 3' sequence is translated into the C-terminal region of the protein, which is responsible for calcineurin binding. Thus, the TVs encode distinct protein isoforms differing from each other only in the N-terminal region (Fig. 16B).
When analyzed by qRT-PCR, DSCR1 reached maximal mRNA level ~60 min after LTD_4-stimulation and declined to baseline level after 6 h (Fig. 17A). However, the probe sets on the U133A array and the primers used for validation do not differentiate between the TVs. Therefore, to identify which TVs are expressed in LTD_4-stimulated HUVECs, transcript variant specific primers were designed. This RT-PCR approach revealed expression of four of the five known TVs, including the TV 6-7-8-9, which has not yet been detected in HUVECs (Fig. 17B).

3.3.3.1. Involvement of NFAT in LTD_4-induced gene expression

Cyclosporin A (CsA) is a widely used immunosuppressive drug, which, via binding to calcineurin, inhibits dephosphorylation of NFAT and its subsequent translocation to the nucleus [Hogan 2003].

A putative alternative promoter sequence of DSCR1 located 5' from exon 5 contains several NFAT binding sites (Fig. 16A) [Harris 2005]. To investigate whether NFAT participates in LTD_4-induced DSCR1 up-regulation, HUVECs were preincubated with 200 ng/ml CsA or

solvent control for 2 h prior to stimulation with LTD_4, and mRNA level of DSCR1 was determined by qRT-PCR (Fig. 17C). $CysLT_2$-R-mediated up-regulation of DSCR1 was completely abolished by CsA. This result confirms that DSCR1 induction by LTD_4 depends solely on NFAT and suggests that the 5-7-8-9 TV, which has NFAT binding sites in its alternative promoter, is the predominantly expressed and regulated DSCR1 isoform.

Upon stimulation with vascular endothelial growth factor (VEGF), NFAT regulates transcription of proinflammatory and proangiogenic genes in ECs such as IL-8, MCP-1, E-Selectin, ICAM-1 (intercellular adhesion molecule 1), NR4A1, NR4A2, NR4A3 and EGR3 [Liu 2003, Minami 2004a]. LTD_4 induced several of these genes, and inhibition of DSCR1 induction by CsA provides indirect evidence for NFAT activation by LTD_4. To study the role of NFAT in LTD_4-triggered gene expression, the mRNA level of several LTD_4-stimulated genes was measured in the presence and absence of CsA (Table 18). Induction of ADAMTS1, CXCL2, EGR3, IL-8, NR4A1, NR4A2 and NR4A3 was reduced, but not diminished in the presence of CsA (Table 18, data above the line). For transcriptional regulation of these genes, additional mechanisms and transcription factors are probably involved in $cysLT_2$-R-mediated up-regulation. By contrast, LTD_4-induced up-regulation of EGR1, E-Selectin and TF was even more pronounced in the presence of CsA (Table 18, data below the line) indicating other regulatory mechanism(s). However, these results need to be confirmed in additional experiments.

Gene	Control	CsA	LTD_4	LTD_4+CsA
ADAMTS1	15.78	13.38	328.69	78.11
CXCL2	2.33	2.43	22.93	15.38
EGR3	0.14	0.15	71.75	17.42
IL-8	4.01	3.15	28.23	14.52
NR4A1	1.49	1.74	224.74	88.25
NR4A2	0.07	0.13	28.92	4.55
NR4A3	0.06	0.05	4.34	1.02
EGR1	37.20	37.14	165.66	662.83
E-Selectin	2.12	2.29	5.68	12.08
TF	4.20	3.51	44.85	90.26

Table 18. Effect of CsA on LTD_4-induced gene induction. HUVECs were preincubated with 200 ng/ml CsA or solvent control for 2 h, subsequently stimulated with 100 nM LTD_4 for 1 h, and mRNA levels were determined by qRT-PCR. Data of a representative experiment are shown as transcript numbers / 10^3 GAPDH.

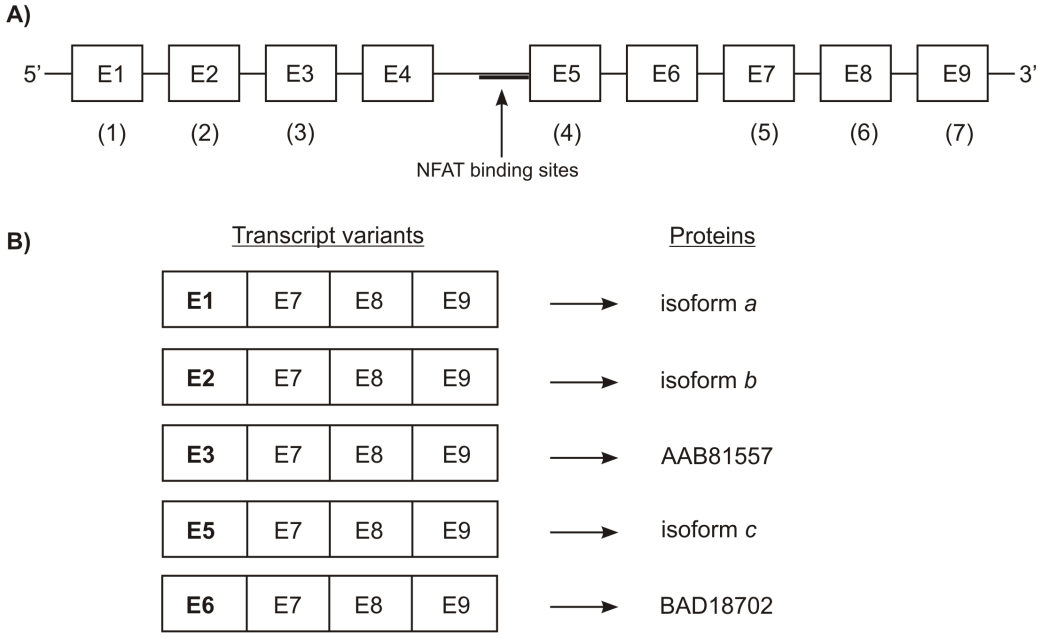

Figure 16. Gene structure and splice variants of DSCR1. A) Exon composition of the DSCR1 genes according to the NCBI databases. Numbers in brackets show exon numbering used in the literature. The arrow marks a region, which contains several binding sites for the transcription factor NFAT and might function as an alternative promoter. **B)** Known transcript variants of DSCR1 and the corresponding proteins.

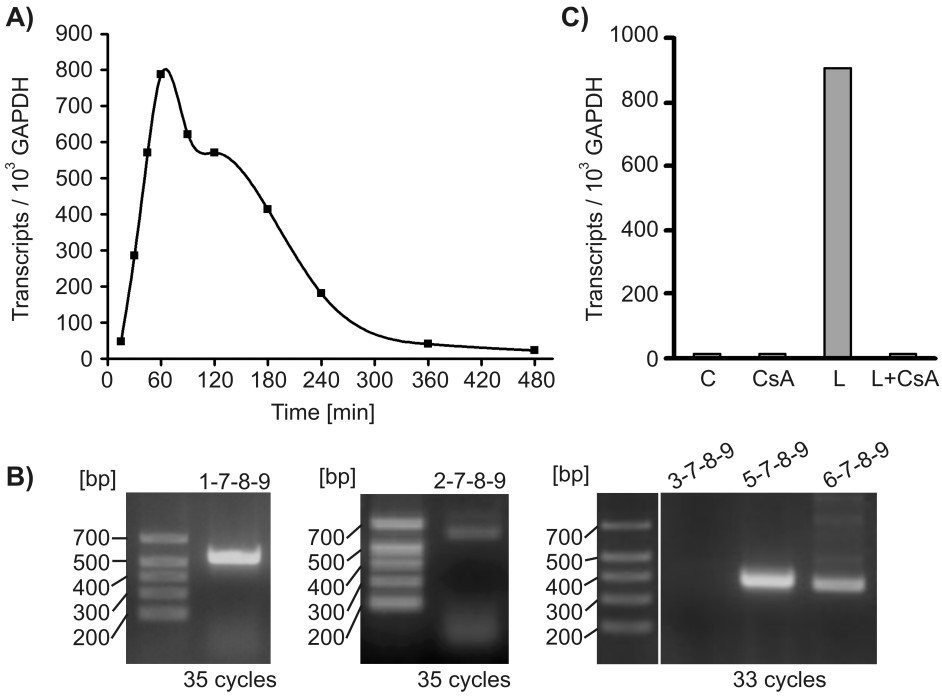

Figure 17. DSCR1 expression in HUVECs. A) DSCR1 kinetics in response to 100 nM LTD_4. **B)** RT-PCR data of DSCR1 transcript variants in LTD_4-stimulated HUVECs. Transcript variant specific primers were used under conditions described in Methods, and cDNA was separated on 2 % agarose gel. **C)** Effect of CsA on LTD_4-induced DSCR1 up-regulation. HUVECs were preincubated with 200 ng/ml CsA for 2 h, then stimulated with 100 nM LTD_4 for 1 h. DSCR1 mRNA was measured by qRT-PCR. Data are representative of three independent experiments. C: control, CsA: cyclosporin A, L: LTD_4, L+CsA: LTD_4 + cyclosporin A

3.2.4. LTD_4 triggers TF expression and cell-associated procoagulant activity

TF is the key initiator of the extrinsic pathway of the blood coagulation cascade [Mackman 2004]. TF is absent on ECs under physiological conditions, but vascular injury or inflammation leads to its expression on the cell surface [Levi 2006].

LTD_4 induced TF mRNA and protein up-regulation in HUVECs. TF mRNA peaked around 60 min and then slowly declined to basal level between 6-8 h (Fig. 18A). TF protein had delayed kinetics compared to the mRNA, with maximal expression at 6 h (Fig. 18B). To determine functional relevance of TF up-regulation by LTD_4, procoagulant activity assay was performed with whole cell lysates. HUVECs were stimulated with 100 nM LTD_4 for 6 h, because maximal protein expression was detected at this time point. Cells were lysed and procoagulant activity was determined by a commercially available kit, which uses recombinant FVIIa, recombinant FX and a specific substrate for FXa. Cell-associated TF procoagulant activity was increased approximately 4-fold in LTD_4-stimulated samples (Fig. 18C).

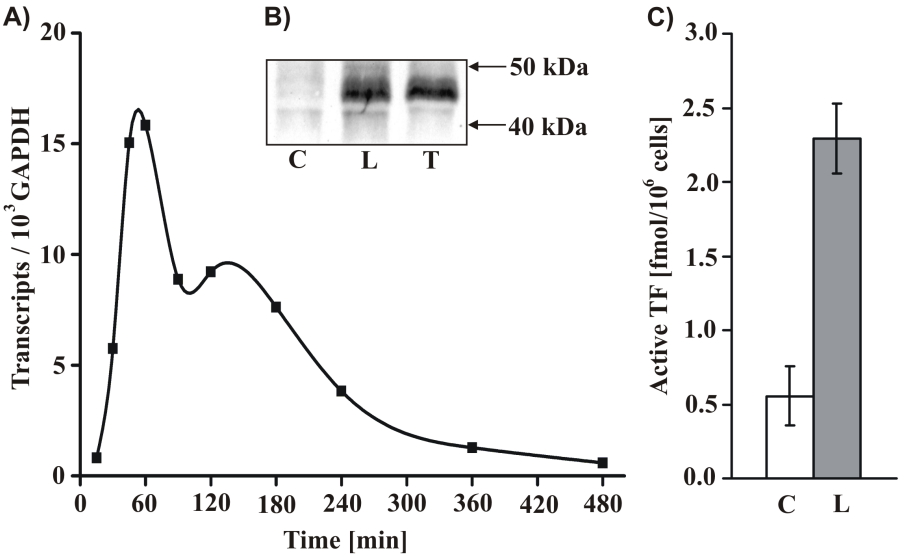

Figure 18. Up-regulation of TF and procoagulant activity by LTD_4. A) LTD_4-induced TF mRNA was measured by qRT-PCR. TF transcripts peaked at ~60 min and reached baseline level at 6 h. **B)** Immunoblot of TF protein 6 h after stimulation with 100 nM LTD_4. 100 U/ml TNFα was used as positive control. Blot is representative of two experiments. **C)** Procoagulant activity of HUVECs at 6 h after LTD_4-stimulation, measured from whole cell lysates. Data are means ± SEM of eight samples derived from three umbilical cord preparations ($p < 0.01$; Student *t* test). C: control, L: LTD_4, T: TNFα

3.3. Late genes regulated by LTD_4

LTD_4 mediates rapid reactions in ECs such as PAF production, PGI_2 release, vWF secretion and P-Selectin surface expression [McIntyre 1986, Cramer 1983, Datta 1995]. In line with literature data, the majority of LTD_4-induced transcriptional changes in ECs were early effects, and gene signatures of later time points showed only a few regulated genes (Fig. 6). Analysis of these data revealed up-regulation of the complement protein CD55 (also called decay accelerating factor for complement) and down-regulation of the $cysLT_2$-R by LTD_4 (Tables 10 and 11 in the Appendix). These two effects were further investigated.

3.3.1. Induction of the complement regulator CD55 by LTD_4

The membrane-bound complement regulators CD55, CD46 (membrane cofactor protein) and CD59 (protectin) are responsible for attenuating complement activation on self surfaces and thus inhibiting complement-mediated lysis of host cells [Brooimans 1992, Janeway 2001]. Vasoactive agonists such as thrombin, histamine and VEGF are known to induce CD55 on endothelial cells [Lidington 2000, Tsuji 1994, Mason 2004].

Microarray data of LTD_4-stimulated HUVECs showed a moderate induction of CD55, but no difference in CD46 and CD59 expression compared to the control (Table 19). To confirm CD55 up-regulation by LTD_4 and to examine its kinetics, CD55 transcript levels were measured by qRT-PCR in LTD_4-stimulated HUVECs. CD55 mRNA peaked at approximately 6 h and stayed elevated during 48 h (Fig. 19A). Expression of CD55 on the cell surface was measured by flow cytometry using a specific antibody. CD55 protein increase appeared 12 h after stimulation and was diminished at 24 h. At 12 h CD55 induction by LTD_4 and thrombin were comparable (Fig. 19B), but the effect of thrombin was still detectable at 24 h (data not shown).

	Affymetrix probe set ID	1 h		6 h		24 h	
		Control	LTD_4	Control	LTD_4	Control	LTD_4
CD55	201925_s_at	1219	2126	1006	2167	1120	1503
	201926_s_at	1307	2393	1342	2645	1329	1822
CD46	207549_x_at	3014	2773	2863	2804	2949	2829
	208783_s_at	2340	2409	2308	2350	2398	2182
CD59	200983_x_at	8944	9014	10009	10499	10103	10940
	200984_s_at	6718	6669	6728	6767	6942	7958
	200985_s_at	6578	6733	6656	7028	6985	7162
	212463_at	2877	2852	3103	3125	2954	3186

Table 19. Signal intensities of the complement regulators CD55, CD46, CD59. All three genes are represented with more than one probe set on the U133A genechip. Every probe set had Present call and detection *p* value <0.065 (not shown). Data are from a single experiment.

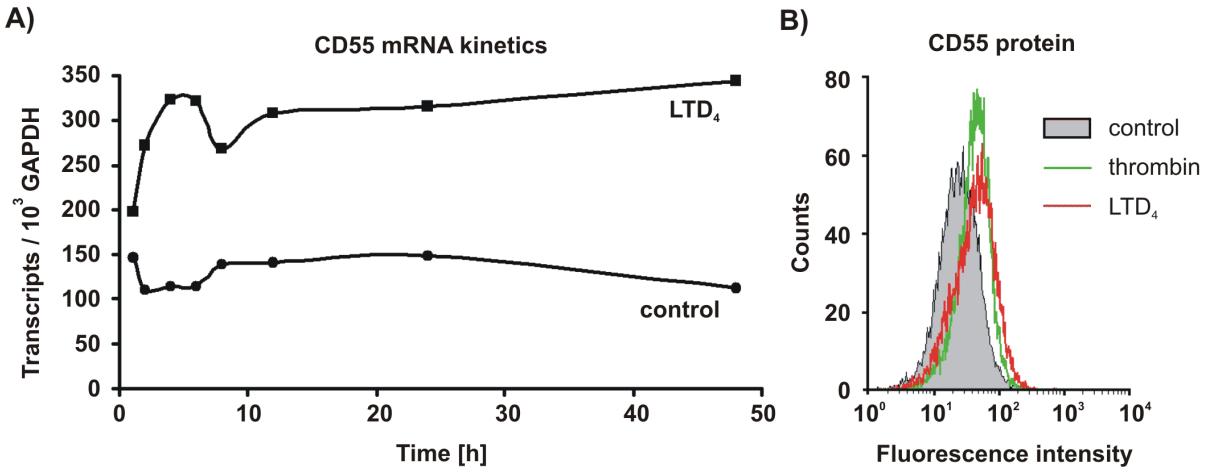

Figure 19. LTD_4-induced up-regulation of the complement inhibitor CD55. A) HUVECs were stimulated with 100 nM LTD_4 for the indicated time points and mRNA kinetics of CD55 was measured by qRT-PCR. Data of a single experiment are shown. **B)** CD55 protein expression on the cell surface was investigated by flow cytometry at 12 h after stimulation. LTD_4-mediated CD55 induction was compared to that triggered by 10 nM thrombin. Data are representative of two independent experiments.

3.3.2. Down-regulation of the cysLT$_2$-R mRNA by LTD$_4$

One of the few genes down-regulated by LTD_4 is its own receptor. Microarray data at 6 h and 24 h showed reduced expression of the $cysLT_2$-R in the LTD_4-stimulated samples (Tables 10 and 11 in the Appendix). mRNA kinetics determined by qRT-PCR revealed rapid $cysLT_2$-R down-regulation with a minimum at 4-6 h after stimulation, which was followed by a long recovery phase. During the monitored interval (48 h), $cysLT_2$-R mRNA did not reach the level measured in unstimulated samples (Fig. 20).

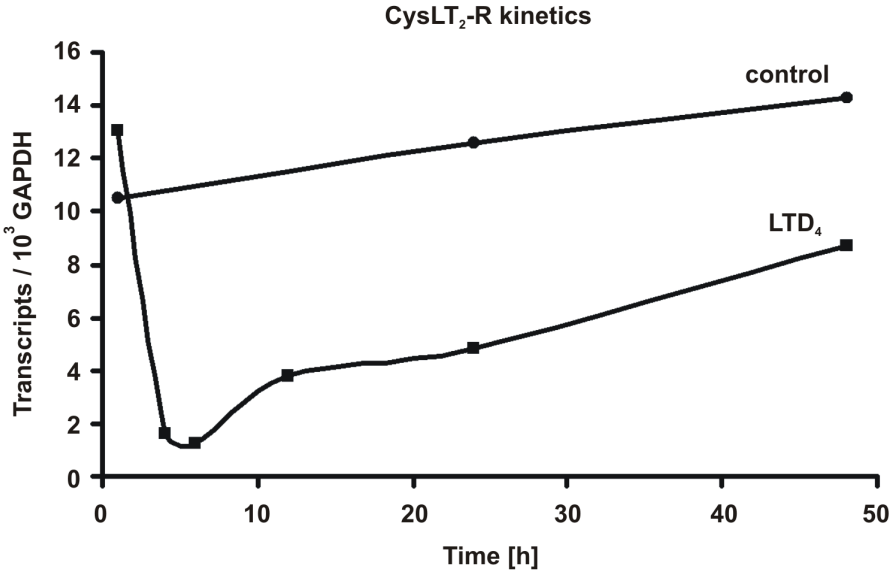

Figure 20. LTD$_4$-mediated down-regulation of cysLT$_2$-R mRNA. HUVECs were stimulated with 100 nM LTD_4 for the indicated time points, and $cysLT_2$-R mRNA levels were determined by qRT-PCR. Results of a single experiment are shown.

Discussion

Data obtained in this work can be summarized as follows: stimulation of ECs with LTD_4 led to a proinflammatory and prothrombotic endothelial phenotype highly resembling that induced by thrombin. The actions of LTD_4 on HUVECs' phenotype include up-regulation of transcription factors involved in vascular biology, chemokine formation and secretion, induction of adhesion molecules and enhancement of TF activity. Up-regulation of potentially important genes that may protect the vascular wall, i.e. DSCR1 and CD55, was also observed. Concomitant activation of $cysLT_2$-R by LTD_4 and PAR-1 by thrombin resulted in enhanced gene expression compared to LTD_4 and thrombin alone.

4.1. Potential role of LTD_4-induced transcription factors in cardio-vascular pathobiology

Activation of the $cysLT_2$-R in HUVECs led to up-regulation of several transcription factors. Among others, members of the EGR, NR4A and KLF transcription factor families were induced by LTD_4 at 1 h as shown by microarray analyses (Fig. 7).

LTD_4 up-regulated three members of the **EGR** family with similar mRNA kinetics (Fig. 12A). Their expression levels, however, were markedly different, which may reflect functional differences of the proteins. EGR1 was the most strongly expressed and induced member, indicating a more important role for EGR1 than for the other two EGRs. EGR2 level was ~100-fold lower than that of EGR1, even when up-regulated by LTD_4. This may be characteristic for EGR2 in vascular cells, since a similarly low level of EGR2 expression in ECs was observed upon treatment with VEGF and thrombin [Liu 2003, Minami 2004a]. EGR3 up-regulation by LTD_4 was higher than that of EGR2, but ~10-fold lower than EGR1. This is comparable to VEGF- and thrombin-induced EGR3 expression in ECs [Liu 2003, Minami 2004a, 2004b]. Even though our data suggest involvement of EGR2 and EGR3 in LTD_4-induced endothelial inflammatory responses, and their presence has also been reported in VEGF- and thrombin-stimulated vessel wall cells [Liu 2003, Minami 2004a, 2004b], their unique role(s) in ECs and vascular biology remains to be determined. The EGRs bind the same

GC-rich element [Silverman 1999], therefore it is difficult to distinguish, which genes are regulated by which EGR member. As EGR2 levels were marginal, the importance of EGR2 in cysLT$_2$-R-triggered endothelial activation is questionable. The relatively strong induction of EGR3 in parallel with EGR1 may implicate their redundant function in LTD$_4$-mediated transcriptional changes, which is supported by the similar induction patterns by other agonists [Liu 2003, Minami 2004b]. Furthermore, the EGR1$^{-/-}$ EGR3$^{-/-}$ double knockout mice die before birth, but both single knockouts, EGR1$^{-/-}$ and EGR3$^{-/-}$, produce live adults [Adamson 2001], pointing to a redundant role of EGR1 and EGR3 *in vivo*.

In contrast to EGR2 and EGR3, EGR1 appears important in vascular biology. LTD$_4$-triggered EGR1 induction was also confirmed at the protein level (Fig. 12B, C). At its highest expression, 1 h after stimulation, EGR1 localized in the nucleus as demonstrated by immunofluorescence. Though the transcriptional activity of EGR1 was not investigated, translocation of EGR1 into the nucleus and up-regulation of genes with EGR-binding sites in their promoter (Table 17) point to a relevant role for EGR1 in LTD$_4$-stimulated endothelial gene expression.

The importance of EGR1 in the vascular system is underlined by a number of reports that show EGR1 expression in vessel wall cells and identify EGR1 target genes involved in vascular biological processes. These genes include PDGF-A, PDGF-B, TF, M-CSF, TGFβ, TNFα, IL-2, p53, uPA (urokinase-type plasminogen activator), ICAM-1, PPARγ (peroxisome proliferative activated receptor γ), VEGF and fibronectin [reviewed in Khachigian 1998 and 2006, Silverman 1999]. In our system several LTD$_4$-induced genes contain EGR binding sites in their promoter (Table 17). In addition to TF, a known EGR1 target gene [Bavendiek 2002, Armesilla 1999, Mechtcheriakova 1999], we found two other genes, for which EGR1-dependent induction was reported in EGR1-transfected HUVECs [Fu 2003]. GEM (GTP binding protein overexpressed in skeletal muscle) and ATF3 (activation transcription factor 3) were strongly up-regulated in our system both by LTD$_4$ and thrombin (Fig. 7). GEM is a member of the small G protein family, which can interact with high-voltage-activated (L-type) Ca^{2+} channels [Béguin 2001]. By inhibiting the L-type Ca^{2+} channel activity, GEM may regulate extracellular Ca^{2+}-triggered processes or signals such as the oscillation in $[Ca^{2+}]_{ic}$ following endothelial cysLT$_2$-R activation [Lötzer 2003]. ATF3, a transcriptional repressor, is implicated in ECs as a survival factor [Kawauchi 2002]. Thus, ATF3 may contribute to the enhanced [^{3}H]-thymidin incorporation observed in LTD$_4$-stimulated HUVECs (unpublished

data C. Kramer, K. Lötzer, A. J. R. Habenicht). Since the promoter region of ATF3 does not contain the consensus EGR binding site, ATF3 might be regulated by EGR1 indirectly, or, alternatively, ATF3 is up-regulated by LTD_4 independent of EGR1.

EGR1 is implicated in hypoxic and ischemic injuries in the vasculature of vital organs such as brain, lung, heart and kidney [reviewed in Khachigian 2006]. EGR1 is expressed at a low level in the arterial wall under physiological conditions but it is rapidly induced by a variety of stimuli and under pathological conditions [reviewed in Khachigian 1998 and 2006, Silverman 1999]. $CysLT_2$-R stimulation by LTD_4 led to EGR1 induction in ECs (Fig. 12), and previous results of other members in our group showed that LTD_4 also up-regulates EGR1 in monocytes via the $cysLT_1$-R (data published in Zhao et al. [2004] and in the NCBI GEO database, Acc. Nr.: GSE1644). Since transcripts of both cysLT-Rs are expressed in atherosclerotic lesions [Spanbroek 2003, Qiu 2006a], our data suggest that cysLTs can contribute to the activated phenotype of plaque forming cells by EGR1 induction. Expression of EGR1 in the vessel wall results in the transcription of proinflammatory and procoagulant genes [McCaffrey 2000, Harja 2004], thus, cysLTs – in concert with other EGR1-inducing agonists – might influence disease progression. However, data on cysLT formation and cell-type specific cysLT-R expression *in vivo* are needed to confirm this hypothesis.

All three members of the **NR4A** transcription factor family were induced by LTD_4 in HUVECs in our experiments (Fig. 13). The orphan nuclear receptors NR4A1 (Nur77 or nerve growth factor-induced B, NGFI-B), NR4A2 (Nurr-1) and NR4A3 (NOR-1) form a subfamily of the steroid / thyroid receptor superfamily [reviewed in Martínez-Gonzalez 2005]. Expression of the three NR4As has been shown in ECs of atherosclerotic lesions, but not of normal artery, by *in situ* hybridization [Arkenbout 2003]. Thus, our *in vitro* data on the induction of the NR4A members by LTD_4 in ECs may connect LTs with pathological states of the vessel wall and suggest a role for them in atherogenesis. However, further investigations are required to determine the *in vivo* relevance of this finding, since LTD_4 is not unique in activation of all three NR4As in ECs; e.g. the potent endothelial mitogen, VEGF, produces a highly similar pattern [Liu 2003].

It is difficult to dissect the actions of individual NR4A proteins, because they bind the same consensus DNA motif and, in addition, may form heterodimers with each other [Martínez-Gonzalez 2005]. The only known NR4A target in ECs is the plasminogen activator inhibitor 1 (PAI-1, SERPINE1). Induction of PAI-1 via NR4A1 in TNFα-stimulated HUVECs, and

colocalization of NR4A1 and PAI-1 proteins in ECs and VSMCs in atherosclerotic coronary artery have recently been demonstrated [Gruber 2003]. Even though both LTD_4 and VEGF up-regulate NR4A1 (and the other two NR4As) in HUVECs, PAI-1 was not induced by LTD_4 (data published in NCBI GEO Acc. Nr.: GSE3589) nor by VEGF [Minami 2004a]. By contrast, thrombin in our experiments up-regulated both NR4A1 and PAI-1 (Tables 9, 12 and 13 in the Appendix). Since we could not show NR4A1 expression at the protein level, we are presently unable to comment further whether the effects of LTD_4 have functional implications for vascular biology. Other NR4A targets in ECs might be some of the LTD_4-induced genes containing the consensus NR4A responsive element in their promoter, such as ADAMTS1, NR4A1 and zinc finger protein 451 (Table 17), several of which are also stimulated by VEGF [Liu 2003, Minami 2004a].

Several members of a third family of transcription factors, namely the **KLFs** were up-regulated by LTD_4 or thrombin. Of the 16 known KLF family members, KLF2, KLF4, KLF5 and KLF6 are implicated in vascular development and pathobiology [reviewed in Suzuki 2005]. Both LTD_4 and thrombin up-regulated KLF2, KLF4 and KLF6 (Fig. 7 and Tables 8, 9 in the Appendix) in HUVECs. KLF2 is restricted to ECs in the vessel wall and thought to be a protective factor in vascular diseases, whereas KLF4 and KLF6 are more widely expressed in the vasculature, but their role in ECs is not characterized [Suzuki 2005].

KLF2 is uniquely and strongly expressed in ECs during embryonic development and in adults under physiological conditions [Suzuki 2005], and can be induced by constitutive laminar shear stress [Dekker 2002]. *In vitro* data on overexpressed KLF2 support the hypothesis that KLF2 counteracts inflammatory and thrombotic processes in ECs [SenBanerjee 2004, Bhattacharya 2005, Lin 2006]. Thus, LTD_4-induced KLF2 may play a role in controlling the proinflammatory endothelial phenotype resulting from $cysLT_2$-R activation. KLFs, similar to the EGR transcription factors, bind GC-rich DNA motifs, however, the sequence specificities for the KLFs have not yet been identified [Suzuki 2005]. It is possible that KLF2 can compete with the EGRs in DNA binding and thus reduce their activity. According to our microarray data, LTD_4 induced KLF2 more strongly than thrombin (Table 7 in the Appendix). This may be responsible for the moderate effect of LTD_4 — in comparison to thrombin — on genes such as E-selectin, vascular cell adhesion molecule 1 (VCAM-1), TF and IL-8 (Fig. 9 and Tables 8, 9, 11 and 12 in the Appendix), all of which showed reduced or inhibited expression in KLF2-transfected HUVECs [SenBanerjee 2004, Bhattacharya 2005, Lin 2006]. Therefore, KLF2

might be part of the feedback mechanisms causing the short term endothelial response to $cysLT_2$-R activation compared to the longer reaction induced by PAR-1 stimulation by thrombin (Fig. 6).

4.2. LTD_4-induced genes in leukocyte recruitment and extracellular matrix rearrangement

Besides up-regulation of transcription factors, LTD_4 stimulation rendered the endothelial phenotype adhesive for platelets and leukocytes. The previously described early transcription-independent effects of LTD_4, such as P-selectin expression, vWF secretion [Datta 1995] and PAF synthesis [McIntyre 1986], are mainly responsible for platelet adhesion and activation and are involved in the initiation of neutrophil attachment. In our experiments, LTD_4 stimulation resulted in induction and secretion of the chemokines IL-8 and CXCL2 (Fig. 14), up-regulation of E-selectin (Fig. 15A) and a moderate VCAM-1 induction (Table 8 in the Appendix). These LTD_4-induced transcriptional changes may implicate LTs in the activation cascade of the attached platelets and leukocytes, initiation of their transmigration as well as attraction of further inflammatory cells to vascular disturbances.

Selectins mediate the first step in leukocyte extravasation and platelet adhesion. In ECs P-selectin is rapidly expressed by releasing the preformed protein from the Weibel-Palade bodies in response to inflammatory stimuli. By contrast, **E-selectin** is present only on activated ECs and its expression requires mRNA and protein synthesis, and thus appears delayed compared to P-selectin [Janeway 2001]. E-selectin is up-regulated in ECs by several stimuli, e.g. proinflammatory cytokines, LPS, lymphotoxin [Bevilacqua 1987], and thrombin [Kaplanski 1997]. Since both E- and P-selectins are important in activated T cell homing into inflamed tissues [reviewed in Ley 2004], LTD_4 may be involved in enhancement of vascular inflammation via induction of selectins on ECs. Deletion of P- and E-selectin in atherosclerotic mouse models results in decreased number of infiltrating monocytes and reduced lesion development [Collins 2000, Dong 1998]. The increased amount of cell adhesion molecules such as ICAM-1, VCAM-1, P-selectin and E-selectin is characteristic for vascular diseases associated with inflammation [Gimbrone 1997, Libby 2002]. Thus, induction of P-selectin expression and up-regulation of E-selectin and VCAM-1 by LTD_4 support a proinflammatory and proatherogen role for LTs in vascular diseases.

Adhesion molecules, although very important for accumulation of leukocytes, are not sufficient to trigger their firm adhesion. To promote leukocyte attachment under flow conditions, chemokines produced by activated ECs are also necessary [Gerszten 1999]. **IL-8** is secreted by ECs in response to numerous stimuli, e.g. LPS, TNFα, IL-1β, VEGF or thrombin [Luster 1998, Minami 2004a, 2004b]. It was first characterized as a potent neutrophil attracting factor [Luster 1998], but recent data show that IL-8 plays a relevant role in monocyte adhesion as well [Gerszten 1999]. Besides recruiting leukocytes, IL-8 is involved in their transmigration through the endothelial monolayer [Lee 2002]. The related chemokine, **CXCL2** (growth-related oncogene β, GROβ or macrophage inflammatory protein 2, MIP2), is a chemotactic factor for PMNs, including basophils [Geiser 1993]. Since stimulation of the endothelial cysLT$_2$-R resulted in up-regulation of IL-8 and CXCL2, our data suggest that LTs can mediate leukocyte attachment on the endothelium and extravasation into the inflamed vessel wall. IL-8 and CXCL2 can also activate the attached PMNs [Geiser 1993], which may result in further LT production. It has been recently demonstrated that IL-8 exerts angiogenic effects on ECs in an autocrine manner via binding to its receptors CXCR1 and CXCR2 [Li 2003]. These two chemokine receptors were expressed but not regulated in our experiments (data published in the NCBI GEO database Acc. Nr.: GSE3589), suggesting an indirect protective effect for endothelial cysLT$_2$-R activation.

Activation of the endothelium and pathological states of the vessel wall are associated with extracellular matrix (ECM) reorganization as cells proliferate, migrate and differentiate. Several types of proteases are involved in these changes of the extracellular content, among them members of a newly described metalloprotease family, the ADAMTS. Its founding member, **ADAMTS1**, was strongly induced by LTD$_4$ in our *in vitro* system (Fig. 15B). ADAMTS1 is present in normal human tissues and throughout the cardiovascular system [Porter 2005, Jönsson-Rylander 2005]. However, it is considered an inflammation-associated gene [Kuno 1997]. Cultured ECs up-regulate ADAMTS1 in response to proinflammatory stimuli [Norata 2004, Minami 2004a]. LTD$_4$ induced ADAMTS1 not only in ECs but, according to data from other members of our group, also in the monocytic cell line MonoMac-6 via the cysLT$_1$-R (data published in the NCBI GEO database Acc. Nr.: GSE1644).
Recently, tissue factor pathway inhibitor 2 (TFPI-2) has been identified as a substrate of ADAMTS1 [Torres-Collado 2006]. TFPI-2 is implicated in the physiological regulation of ECM degradation and reorganization as an inhibitor of plasmin, trypsin, kallikrein, FXa and the TF-FVIIa complex [Chand 2005]. Versican, another substrate of ADAMTS1 [Sandy 2001],

is abundantly present in normal vessel wall, whereas human aortic aneurysm is associated with decreased versican levels [reviewed in Wight 2004]. Thus, LTD_4-induced ADAMTS1 may lead to imbalanced ECM remodeling, which accompanies vascular disorders. LTs have already been implicated in the pathogenesis of aortic aneurysm [Zhao 2004]. Apolipoprotein E $(ApoE)^{-/-}$ $5\text{-}LO^{-/-}$ mice show reduced aortic aneurysm formation and matrix metalloprotease activity compared to $ApoE^{-/-}$, suggesting an indirect effect of the 5-LO pathway on ECM degrading enzymes. Up-regulation of ADAMTS1 by LTD_4 indicate novel mechanisms of LT actions in vascular diseases.

Another aspect of ADAMTS1 function is that it can exert anti-angiogenic effects on ECs. When bound to the EC surface via CD36, ADAMTS1 prevents VEGF-VEGFR interaction [Luque 2003]. This raises the possibility that LTs could have protective rather than injurious effects. Thus, via induction of ADAMTS1 secretion, LTs might negatively regulate intimal neovascularisation — a process necessary for plaque growth or neointima formation in states of vascular diseases.

4.3. Procoagulant EC phenotype evoked by LTD_4

Stimulation of HUVECs with LTD_4 resulted in the up-regulation of **TF** mRNA, protein and cell-associated procoagulant activity (Fig. 18). ECs do not express TF under physiological conditions [Wilcox 1989], but TF can be induced by tissue injury or by various agonists such as TNFα, IL-1β, CD40 ligand, serotonin, histamine, thrombin, oxidized low density lipoprotein (LDL), VEGF [Camera 1999, Bavendiek 2002, reviewed in Steffel 2006]. Signaling pathways of the diverse agonists that induce TF in HUVECs converge on four transcription factors, namely EGR1, NFAT, nuclear factor κ B (NFκB) and activator protein 1 (AP-1) [Moll 1995, Bavendiek 2002, Armesilla 1999, Mechtcheriakova 1999].
Induction and nuclear translocation of EGR1 by LTD_4 was proved (Fig. 12). Although direct binding of LTD_4-induced EGR1 to the TF promoter was not investigated, EGR1 may mediate LTD_4-induced TF transcription. This is supported by the facts that *i)* EGR1 up-regulation precedes the TF mRNA peak (Figures 12A and 18A), and *ii)* EGR1 protein is present in unstimulated HUVECs (Fig. 12B), which can explain that TF is an immediate-early gene in response to LTD_4 in our system (Fig. 10).

The role of NFAT in LTD_4-induced TF up-regulation does not seem relevant, since inhibition of NFAT with CsA induced TF mRNA even more strongly than LTD_4 alone (Table 18). That this is not uncommon for inflammatory stimuli, is supported by the results of Hölschermann *et al.* (2001), who showed similar effect for LPS, TNFα and ionomycin in CsA-treated HUVECs. Cooperation between transcription factors on TF induction in HUVECs has also been described. TF stimulation by VEGF [Armesilla 1999, Mechtcheriakova 1999] or by an oxidized phospholipid component of oxidized LDL [Bochkov 2002] requires both NFAT and EGR1, but in contrast to our data with LTD_4, blocking of NFAT by CsA results in reduced TF expression and activity. This further strengthens the hypothesis that up-regulation of TF by LTD_4 is independent of NFAT.
Although thrombin-induced transcriptional changes involve NFκB [Minami 2004b] and our data revealed high similarities between the LTD_4- and thrombin-activated gene signatures, translocation of NFκB could not be shown in ECs in response to LTD_4 by immuno-histochemistry (unpublished data, S. Jahn, K. Lötzer & A. J. R. Habenicht).
Thus, our data suggest that LTD_4-triggered TF induction is mediated by EGR1 and does not require NFκB or NFAT. The fact that the mechanisms of TF up-regulation in response to LTD_4 differ from those induced by thrombin and VEGF supports a distinct role for cysLTs and the $cysLT_2$-R in EC biology.

Up-regulation of TF mRNA by LTD_4 was followed by TF protein expression with a maximum at 6 h after stimulation (Fig. 18B). For LTD_4-induced TF protein detection whole cell lysates were used, but in addition to the cellular TF protein, TF can be released from ECs either in the form of microparticles [Mallat 2000] or as alternatively spliced soluble TF [Szotowski 2005]. Since the primer pair used in this work (Table 4) does not differentiate between the membrane bound and soluble isoforms and the amount of released TF protein was not determined, our data do not necessarily correlate with the total amount of the LTD_4-induced TF protein, and the effects of LTD_4 are not directly comparable with those of TNFα.

ECs, though synthesize TF protein in response to a number of stimuli, do not express it immediately on the cell surface [Camera 1999, Hölschermann 2001]. TF protein is distributed in three cellular pools: surface (active) TF, cytoplasmic TF, and latent or encrypted TF [Schecter 1997, Steffel 2006]. In addition, surface or surface-associated proteins can prevent TF binding to FVII/VIIa [Bhattacharjee 2005]. Therefore, our aim was to investigate whether LTD_4-mediated TF up-regulation may trigger procoagulant activity. Using a specific system

that mimicks the extrinsic coagulation pathway (Fig. 2), we could show that the LTD_4-induced increase in TF protein was accompanied by TF-dependent procoagulant activity of the cells. Though these results point to the $cysLT_2$-R-mediated induction of active TF, further investigations are needed to determine the cellular distribution of TF and the mechanism(s) required for its surface appearance.

Independent of its cellular distribution, up-regulated TF is a marker for disturbed hemostatic balance of ECs and has been implicated in the pathogenesis of multiple vascular disorders such as ischemia / reperfusion injury [Erlich 2000, Mackman 2003], thrombosis [Mackman 2004], atherosclerosis [Steffel 2006] or disseminated intravascular coagulation [Levi 2006]. Our data suggest that cysLTs increase the active TF pool in ECs, and thus, LTs may contribute *in vivo* to the prothrombotic state of the endothelium during vascular diseases in concert with other agonists from the inflammatory microenvironment.

4.4. LTD_4-induced CD55 may protect ECs against enhanced complement activation

The complement system is an effector component of the innate immune response, and consists of inactive soluble proteins as well as soluble and membrane-bound regulators. The mechanism of complement activation is similar to that of the coagulation cascade [Janeway 2001], and cross-talk between the two systems has been demonstrated [Huber-Lang 2006]. Complement activation is enhanced during inflammation. To prevent tissue damage caused by complement-mediated lysis, host cells, especially those in contact with blood, express several regulator molecules [Janeway 2001]. ECs constitutively express the complement regulators CD46, CD55 and CD59 [Brooimans 1992].

Among the few genes regulated by LTD_4 at later time points is the complement regulator **CD55** with a maximal fold change of ~3 (Fig. 19A and Table 19). LTD_4-triggered CD55 up-regulation was moderate at the protein level (Fig. 19B), though similar to numerous other agonists that stimulate CD55 in ECs, such as TNFα, IFNγ [Mason 1999], VEGF [Mason 2004], histamine [Tsuji 1994], or thrombin [Lidington 2000]. CD55 inhibits the complement cascade at the level of the central component, C3, by inactivating the C3 and C5 convertases

[Fujita 1987]. Since the efficiency of the complement system is built on the amplifying activation mechanism, a small change in regulator protein amount is sufficient to reduce complement activity. LTD_4-induced CD55 therefore provides enhanced protection for ECs against autologous complement attack. Furthermore, attenuating the complement cascade reduces liberation of the vasoactive anaphylatoxins, C3a and C5a [Janeway 2001, Schraufstatter 2002].

In addition to its increased surface expression in response to a number of agonists, CD55 can be released into the environment and accumulate in the ECM [Tsuji 1994, Hindmarsh 1998]. Shedding and deposition in the ECM is induced only by stimuli that activate PKC, i.e. phorbol myristate acetate, wheat germ agglutinin or histamine, but not by other potent EC agonists such as TNFα, IL-1β and IL-4 [Tsuji 1994, Hindmarsh 1998]. Although LTD_4-dependent CD55 release was not investigated here, the fact that cysLT_2-R stimulation increases $[Ca^{2+}]_{ic}$ leading to PKC activation (unpublished data C. Kramer, K. Lötzer & A. J. R. Habenicht), suggests that LTD_4 may also enhance CD55 shedding and secretion. Moreover, CD55 showed elevated mRNA levels for 48 h (Fig. 19A), whereas CD55 protein expression returned to baseline levels by 24 h. This hints to CD55 release, which could explain the discrepancies between the induction of CD55 mRNA and surface protein in response to LTD_4.

4.5. Negative feedback mechanisms of cysLT_2-R activation may involve DSCR1

One of the most strongly up-regulated genes in LTD_4-stimulated HUVECs was **DSCR1** (Figures 9 and 17). The DSCR1 protein (also called calcipressin 1, modulatory calcineurin-interacting protein 1, regulator of calcineurin, or Adapt78) is involved in protection against oxidative and calcium-mediated stress [Ermak 2002]. Stimuli causing increased $[Ca^{2+}]_{ic}$ activate the calcineurin–NFAT system, and among other inflammatory genes, up-regulate DSCR1 [Liu 2003, Minami 2004a, Yao 2004]. DSCR1, in turn, blocks calcineurin and the NFAT-dependent pathway, forming a negative feedback loop [Hesser 2004, Iizuka 2004].

DSCR1 has several TVs due to alternative splicing and a putative alternative promoter [Harris 2005]. Four of the five known TVs were detected in LTD_4-stimulated HUVECs (Fig. 17B). This confirms data of others on endothelial expression of the TVs with exon composition

1-7-8-9, 2-7-8-9 and 5-7-8-9. Furthermore, it shows for the first time the presence of the TV 6-7-8-9 in ECs. Since the protein isoforms corresponding to the various TVs have the same C-terminal part responsible for calcineurin binding, they may have the same function. The variable N-terminal domain may be involved in regulation or interaction with other proteins but its importance in ECs has not yet been clarified. The most strongly up-regulated TV by LTD_4, containing the exons 5-7-8-9, is the one also induced by thrombin and VEGF in ECs [Minami 2004a]. Thus, this DSCR1 isoform may have a similar function in controlling signaling pathways of $cysLT_2$-R, PAR-1 and the VEGF receptor KDR (kinase insert domain receptor).

To study the possible role of the endogenous calcineurin inhibitor DSCR1 in LTD_4-dependent EC activation, we used the exogenous calcineurin inhibitor CsA. Preincubation of HUVECs with CsA and subsequent LTD_4-stimulation resulted in lower expression of inflammatory genes, such as IL-8, CXCL2, ADAMTS1, NR4A1, NR4A2, NR4A3 and EGR3 (Table 18). This indicates involvement of the Ca^{2+}–calcineurin–NFAT pathway in $cysLT_2$-R-mediated transcriptional changes. Thus, NFAT-mediated transcription of these genes in LTD_4-stimulated HUVECs may be repressed via the concomitantly synthesized DSCR1. This hypothesis is in line with literature data. Blocking the calcineurin–NFAT pathway in ECs by CsA addition [Liu 2003] or DSCR1 overexpression [Minami 2004a] resulted in lower induction of proinflammatory and proangiogenic genes (e.g. NR4A1, NR4A2, NR4A3, EGR3, IL-8, E-selectin) and higher induction of ADAMTS1 after stimulation with thrombin or VEGF. In our experiments, the majority of the LTD_4-induced genes were regulated similarly, with the exception of ADAMTS1 and E-selectin. When the calcineurin–NFAT pathway was blocked by CsA and HUVECs were stimulated with LTD_4, ADAMTS1 expression was strongly reduced, suggesting a role for NFAT, whereas E-selectin transcription did not seem to require NFAT activity as its expression was higher than in untreated cells (Table 18). These data point to a general negative regulation of inflammatory genes via DSCR1 in ECs with some agonist-dependent differences.

LTD_4 (100 nM) induced DSCR1 more strongly than thrombin (10 nM) (Fig. 9) or VEGF (25 ng/ml; control: 70, LTD_4: 2,234, VEGF: 906 transcripts / 1,000 GAPDH measured by qRT-PCR), and the majority of the genes examined were regulated through NFAT (Table 18), suggesting that the relatively short term effects of LTD_4 on ECs are due to the action of

DSCR1. Although VEGF, thrombin and LTD_4 trigger highly similar gene signatures at 1 h, the differences in signaling pathways and in gene expression intensities strongly influence the response of ECs to the given stimuli.

4.6. LTD_4 stimulation leads to cysLT$_2$-R down-regulation in ECs

Besides desensitization, internalization and recycling, which are characteristic of most GPCRs, transcriptional and translational changes may also contribute to receptor availability. There is no published data on the kinetics and mechanisms of **cysLT$_2$-R** desensitization, and only few studies investigated cysLT$_2$-R mRNA or protein regulation [Mellor 2003, Lötzer 2003, Sjöström 2003]. In our experiments, LTD_4 had a negative feedback effect on cysLT$_2$-R. Stimulation with LTD_4 led to strong decrease in cysLT$_2$-R mRNA (Fig. 20, Tables 10 and 11 in the Appendix). Though cysLT$_2$-R protein was not directly measured, functional surface-expressed cysLT$_2$-R was also reduced, as indicated by the lack of $[Ca^{2+}]_{ic}$ increase in response to repeated LTD_4 addition: HUVECs stimulated with 100 nM LTD_4 did not produce Ca^{2+} signals at 1 h, 6 h or 24 h after the first LTD_4 stimulus, even if the culture medium was replaced to remove LTD_4 after the first stimulation (unpublished data, C. Kramer, K. Lötzer & A. J. R. Habenicht). Via reducing cysLT$_2$-R expression, cysLTs may control EC activation and prevent escalation of the inflammatory response. This receptor down-regulation, however, is not a general LT attribute, since LTB_4 up-regulates BLT_1-R and has no effect on BLT_2-R in HUVECs [Qiu 2006b].

4.7. Comparison of LTD_4- and thrombin-induced gene expression patterns

Endothelial cysLT$_2$-R-activated genes gave rise to a proinflammatory and prothrombotic phenotype. These early gene signatures of LTD_4 resembled those of thrombin determined in parallel (Figures 6 and 7). In addition to previously described shared activities of the two agonists, their gene expression patterns at 1 h after stimulation showed high similarity, and significantly up-regulated genes at this time point strongly correlated. The two later time points, i.e. 6 h and 24 h, did not show this uniformity since the effects of LTD_4 were less potent than those of thrombin. This might be explained by the strong counter-regulatory mechanisms

activated by LTD_4 including the feedback loop with the calcineurin inhibitor DSCR1 and the down-regulation of $cysLT_2$-R. Although attenuation of the Ca^{2+}–calcineurin–NFAT pathway via DSCR1 is also triggered by PAR-1 activation [Minami 2004a], based on the stronger induction of DSCR1 by LTD_4 (Fig. 9), our data suggest a more powerful inhibition in the case of $cysLT_2$-R activation. Furthermore, thrombin responsiveness can be restored in a shorter time due to the pre-synthesized PAR-1 pool [Ellis 1999] and unchanged PAR-1 transcript levels (data published in NCBI GEO Acc. Nr.: GSE3589).

Other differences between thrombin and LTD_4 can be seen when looking at the kinetics of the activated genes. LTD_4-induced transcripts showed an early peak and returned to baseline level soon after stimulation, whereas several thrombin stimulated genes reached their mRNA maximum later and stayed at an elevated level for longer periods of time. Thrombin induced IL-8 and E-selectin with prolonged kinetics (with a maximum at ~4 h) [Kaplanski 1997] compared to those induced by LTD_4 (Figures 14A and 15A). Up-regulation of VCAM-1 by LTD_4 already appeared at 1 h (Table 8 in the Appendix), whereas in the case of thrombin it became apparent after 4 h (Table 12 and [Minami 2004b]). These data suggest similar but not identical actions for LTD_4 and thrombin. $CysLT_2$-R stimulation caused early and rapid changes in the endothelium, and PAR-1 activation by thrombin led to a later and somewhat longer response, which may reflect distinct biological roles for these receptors in vascular inflammation.

The fact that stimulation of $cysLT_2$-R or PAR-1 led to the activation of nearly the same genes may be an indication of similar G protein utilization. Endothelial PAR-1 can interact with multiple types of G proteins such as $G_{i/o}$, G_q, $G_{12/13}$ [Gilchrist 2001, Vanhauwe 2002], whereas no data is published on endothelial $cysLT_2$-R. The increase in $[Ca^{2+}]_{ic}$ in response to LTD_4 stimulation [Lötzer 2003] as well as the NFAT-mediated up-regulation of DSCR1 and other genes point to the involvement of G_q, but additional G proteins may also mediate signals from the $cysLT_2$-R.

That the gene signatures induced by LTD_4 and thrombin are not the results of general G protein activation, is supported by the analysis of VEGF-induced gene signatures [Minami 2004a, Liu 2003, Abe 2001]. Early genes induced by activation of the high affinity VEGF receptor, KDR, a receptor tyrosine kinase, show high similarity to those induced by thrombin [Minami 2004a]. Since none of these microarray datasets are publicly available and we did not perform microarray experiments with VEGF-stimulated HUVECs, a complete and direct comparison

with our data is not possible. Still, the majority of the most strongly induced early genes by LTD_4 and VEGF are identical (Table 20). In the study from Liu et al. (2003) the same microarray technology and genechip type was used as in our experiments, more than two samples per group were analyzed, and fold change values are published. Therefore, it was best fit for showing the likeness between VEGF and LTD_4. VEGF is a potent endothelial mitogen and survival factor [Ferrara 2001], and thrombin induces endothelial proliferation and migration [Tsopanoglou 2004, Minami 2004b]. The high similarity of VEGF's and thrombin's early gene signatures and their divergence from those of other agonists such as TNFα [Minami 2004a] might indicate "angiogenic gene expression patterns". Since LTD_4-induced gene signatures resemble those of thrombin and VEGF, our data are consistent with the hypothesis that LTD_4 may act as an angiogenic factor. This is supported by studies reporting angiogenic effects of LTD_4 such as increased collagenous protein biosynthesis and vascular density in the chick corioallantoic membrane [Tsopanoglou 1994], and induction of HUVEC proliferation [Fierro 2002]. Endothelial $cysLT_2$-R-mediated angiogenic processes might be relevant in pathological angiogenesis during vascular diseases or in wound healing and neoangiogenesis

Gene	VEGF		LTD_4
	45 min	90 min	60 min
ADAMTS1	4	3	9
DSCR1	8	10	16
HBEGF	3	3	7
DUSP1		5	5
DUSP5	3	3	2
EGR2	6		9
EGR3	10	10	22
F3	19		17
IL8	7	3	8
KCNJ2	3	3	3
MAP3K8	4	9	6
NR4A1	8	5	33
NR4A2	12	17	47
NR4A3		7	26
PTGS2	3	4	15
STC1		4	2

Table 20. The most strongly induced genes by VEGF and LTD_4. The columns show fold changes in gene expression after VEGF [Liu 2003] or LTD_4 stimulation. In case of VEGF, data represent the result of three microarray experiments (for details see Liu 2003), and for LTD_4 four datasets were analyzed. HBEGF: heparin-binding EGF-like growth factor; DUSP1: dual specificity phosphatase 1; DUSP5: dual specificity phosphatase 5; F3: tissue factor; KCNJ2: potassium inwardly-rectifying channel, subfamily J, member 2; MAP3K8: mitogen activated protein kinase kinase kinase 8; PTGS2: cyclooxygenase 2; STC1: stanniocalcin 1

after vascular injury. The inflammatory reaction of the injured vessel wall can trigger the production and release of progenitor cells from the bone marrow and other tissues [reviewed in Goldschmidt-Clermont 2005]. Homing, anchorage and integration of these progenitors depend on microenvironmental effects, where locally produced agonists including LTs may play an important role.

Treatment of ECs with LTD_4 and thrombin together resulted in enhanced gene expression compared to either agonist alone (Fig. 8, Table 14 in the Appendix). These data suggest that concomitant stimulation of the endothelial cysLT_2-R and PAR-1 may augment the inflammatory response of ECs. This may be relevant *in vivo* during inflammatory states in the vasculature, e.g. thrombus formation or atherosclerotic plaque disruption when conversion of prothrombin to thrombin and production of LTs directly by leukocytes or via transcellular metabolism by platelets and ECs are simultaneously initiated [Maclouf 1989, Qiu 2006a, Cirino 1996].

4.8. Conclusions

This study aimed to understand transcriptional and selected tranlational events resulting from activation of the cysLT_2-R in ECs and to interpret these effects in the context of vascular biology. The diversity of LTD_4-induced gene signatures points to a comprehensive early proinflammatory and prothrombotic EC phenotype. In addition to identifying genes responsible for the inflammatory actions of LTs, the analysis of LTD_4-triggered gene signatures revealed genes and regulatory mechanisms that can control EC activation and thus limit inflammation. Investigating cysLT-induced gene expression patterns in ECs shed more light on cysLT_2-R actions in a non-transfectant system. These data may provide insight into the mechanisms of LT effects in vascular disorders.

Summary

LTs, i.e. LTB_4 and the cysLTs, are lipid mediators with potent biological activities. LTs are generated either endogenously by activated leukocytes from arachidonic acid in response to inflammatory stimuli or via transcellular metabolism from leukocyte-derived LTA_4. LTs are viewed mainly as inflammatory mediators, thereby contributing to a variety of disease conditions. They have many biological effects, the most prominent of which are chemotactic, vasoactive and smooth muscle contracting activities. CysLT actions are mediated by three cysLT-Rs that are differentially expressed on leukocytes and on blood vessel wall cells. The $cysLT_2$-R is selectively expressed by ECs, implying functions in the cardiovascular system.

To delineate $cysLT_2$-R-mediated transcriptional changes in ECs, HUVECs were stimulated with LTD_4 and gene signatures were determined by microarray analyses. Thrombin, the potent vasoactive agonist, was used for comparison. LTD_4 led to an early proinflammatory and prothrombotic gene expression pattern highly resembling that induced by thrombin activation of PAR-1. When added together, LTD_4 and thrombin generally enhanced gene expression. The most strongly induced genes by LTD_4 included transcription factors of the EGR and NR4A families, CXC chemokines, intracellular regulators such as DSCR1, adhesion molecules such as E-selectin, the metalloprotease ADAMTS1, the blood coagulation initiator TF, and the complement regulator CD55. Induction of these genes was confirmed and their mRNA kinetics was determined by qRT-PCR. LTD_4 stimulation also resulted in up-regulation of EGR1 protein and its translocation to the nucleus, IL-8 protein secretion into the culture medium, TF expression and TF-dependent cell-associated procoagulant activity as well as increased amounts of cell surface CD55.

This study is the first to characterize $cysLT_2$-R-triggered endothelial gene signatures. It provides evidence for the potential transcription- and translation-dependent inflammatory actions of cysLTs in the vasculature *in vivo*.

Zusammenfassung

Leukotriene (LTe), d.h. LTB_4 und die Cysteinylleukotriene (cysLTe) LTC_4, LTD_4 und LTE_4, sind hochaktive Lipidmediatoren. LTe werden entweder von aktivierten Leukozyten nach inflammatorischen Stimuli endogen aus Arachidonsäure synthetisiert oder durch transzelluläre Metabolisierung des von Leukozyten stammenden LTA_4 produziert. LTe sind als Mediatoren inflammatorischer Reaktionen bekannt und werden mit verschiedenen Erkrankungen in Zusammenhang gebracht. LTe haben viele biologische Effekte, von denen die bedeutendsten die chemotaktischen und vasoaktiven Wirkungen sowie der Einfluß auf die Kontraktion glatter Muskelzellen sind. Ihre Funktionen üben CysLTe durch drei CysLT-Rezeptoren (CysLT-Ren) aus, die von Blutleukozyten und Gefäßwandzellen differentiell exprimiert werden. Endothelzellen (ECs) exprimieren selektiv den $CysLT_2$-R, was auf eine Funktion im kardiovaskulären System hindeutet.

Um die $CysLT_2$-R-initiierten transkriptionellen Änderungen in ECs zu verfolgen wurden HUVECs (ECs aus der humanen Umbilikalvene) mit LTD_4 stimuliert und die Gensignaturen mittels Microarrayanalysen untersucht. Thrombin, das als Prototyp vasoaktiver Agonisten in HUVECs gilt, wurde zum Vergleich genutzt. LTD_4 induzierte ein proinflammatorisches und prothrombotisches Genexpressionsmuster, welches große Ähnlichkeiten zu dem von Thrombin über die Aktivierung des PAR-1 induzierten Muster zeigte. Die kombinierte Stimulation mit LTD_4 und Thrombin ergab eine verstärkte Genexpression. Zu den am stärksten aktivierten Genen von LTD_4 gehörten Transkriptionsfaktoren der Familien EGR und NR4A, CXC Chemokine, intrazelluläre Regulatoren wie DSCR1, Adhäsionsmoleküle wie E-Selektin, die Metalloprotease ADAMTS1, der Initiator der Gerinnungskaskade TF (Gewebsthromboplastin) und der Komplementregulator CD55. Quantitative RT-PCR Analysen bestätigten die Induktion dieser Gene und klärten ihre mRNA Kinetik auf. Die Inkubation mit LTD_4 führte ebenfalls zur Hochregulation des EGR1 Proteins und dessen Translokation in den Zellkern, der Sekretion des IL-8 Proteins in das Kulturmedium, der Expression von TF Protein und der davon abhängigen prothrombotischen Aktivität der Zellen sowie der erhöhten Expression des CD55 an der Zelloberfläche.

Die vorliegende Arbeit charakterisierte endotheliale Gensignaturen des $CysLT_2$-Rs. Die Daten geben somit Hinweise auf potentielle transkriptions- und translationsabhängige inflammatorische Wirkungen der CysLTe im kardiovaskulären System *in vivo*.

Appendix

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Abbreviations

5-LO	5-lipoxygenase
AA	arachidonic acid
ADAMTS1	a disintegrin-like and metalloproteinase with thrombospondin motif 1, type 1
AT	Annealing temperature
ATF3	activation transcription factor 3
BLT-R	LTB_4 receptor
$[Ca^{2+}]_{ic}$	intracellular Ca^{2+} concentration
COX-2	cyclooxygenase 2
CsA	cycloporin A
CXCL2	CXC chemokine ligand 2
cysLT	cysteinyl leukotriene
cysLT-R	cysteinyl leukotriene receptor
DC	dendritic cell
DSCR1	Down syndrome critical region gene 1
EC	endothelial cell
ECM	extracellular matrix
EGR	early growth response
F	coagulation factor
FLAP	5-lipoxygenase activating protein
GAPDH	glycerine aldehyde-3 phosphate dehydrogenase
GEM	GTP binding protein overexpressed in skeletal muscle
GPCR	G protein coupled receptor
HUVEC	human umbilical vein endothelial cell
ICAM	intercellular adhesion molecule
IL	interleukin
KDR	kinase insert domain receptor
KLF	Krüppel-like factor
LDL	low density lipoprotein
LPS	lipopolysaccharide
LT	leukotriene
MCP-1	monocyte chemoattractant protein 1
NCBI	National Center for Biotechnology Information
NFAT	nuclear factor of activate T cells
NFκB	nuclear factor κ B

NR4A	nuclear receptor subfamily 4, group A
PAF	platelet-activating factor
PAI	plasminogen activator inhibitor
PAR	protease activated receptor
PGI_2	prostacyclin
PKC	protein kinase C
PMN	polymorphonuclear cell
SF	scaling factor
TF	tissue factor
TGF	tumor growth factor
TNF	tumor necrosis factor
TV	transcript variant
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell
vWF	von Willebrand factor

Supplementary tables

Table 7. Signal intensity values of 37 genes up-regulated by LTD_4 as displayed in Fig. 9. Columns show signal intensity values without normalization.

Affymetrix probe set ID	Gene title	Gene symbol	Control				LTD_4				Thrombin			
			1	2	3	4	1	2	3	4	1	2	3	4
200632_s_at	N-myc downstream regulated gene 1	NDRG1	1228	1604	1253	1234	2556	4199	4990	2905	1537	1873	5324	2876
208078_s_at	SNF1-like kinase	SNF1LK	501	446	210	477	969	2812	3467	1347	538	823	5645	1509
219371_s_at	Kruppel-like factor 2 (lung)	KLF2	1034	1284	810	1328	2684	4258	3037	1746	1235	2196	5401	2194
221841_s_at	Kruppel-like factor 4 (gut)	KLF4	244	391	634	428	727	2092	3032	740	579	1612	6120	2057
201041_s_at	dual specificity phosphatase 1	DUSP1	1600	1993	1762	1603	5020	11782	9382	5228	5068	6151	11030	7007
212614_at	AT rich interactive domain 5B (MRF1-like)	ARID5B	415	494	406	555	1561	2457	1979	1318	1662	1613	2495	1440
201531_at	zinc finger protein 36, C3H type, homolog (mouse)	ZFP36	321	358	311	190	1124	5220	2724	1983	1757	2037	2435	2629
210764_s_at	cysteine-rich, angiogenic inducer, 61	CYR61	1273	947	727	679	5102	13985	8499	5249	12498	16029	14958	12197
203821_at	heparin-binding EGF-like growth factor	HBEGF	446	236	186	396	1661	2768	2840	2184	2320	1772	5184	3718
204748_at	prostaglandin-endoperoxide synthase 2	PTGS2	94	301	366	366	1280	7185	4429	3452	707	1170	3789	2847

Affymetrix probe set ID	Gene title	Gene symbol	Control				LTD_4				Thrombin			
			1	2	3	4	1	2	3	4	1	2	3	4
200632_s_at	N-myc downstream regulated gene 1 (prostaglandin G/H synthase and cyclooxygenase)	NDRG1	1228	1604	1253	1234	2556	4199	4990	2905	1537	1873	5324	2876
205249_at	early growth response 2 (Krox-20 homolog, Drosophila)	EGR2	70	122	64	111	409	1196	976	863	355	244	1382	592
202150_s_at	neural precursor cell expressed, developmentally down-regulated 9	NEDD9	918	917	348	450	2244	2272	2429	1193	2594	1828	3074	2271
218880_at	FOS-like antigen 2	FOSL2	286	349	93	241	774	703	1117	419	840	396	1322	561
206765_at	potassium inwardly-rectifying channel, subfamily J, member 2	KCNJ2	971	727	661	865	2533	3134	2832	1948	1821	1545	2393	1981
214438_at	H2.0-like homeo box 1 (Drosophila)	HLX1	742	641	386	524	2250	3120	2389	1455	1818	1701	2169	1185
221031_s_at	hypothetical protein DKFZp434F0318	DKFZP434F0318	1029	937	259	453	2450	3734	2555	1671	1924	1430	3513	1685
214508_x_at	cAMP responsive element modulator	CREM	570	560	437	642	1622	1520	1584	824	908	612	1692	760
207980_s_at	Cbp/p300-interacting	CITED2	345	217	448	370	1246	897	810	714	738	858	1513	726

Affymetrix probe set ID	Gene title	Gene symbol	Control				LTD_4				Thrombin			
			1	2	3	4	1	2	3	4	1	2	3	4
200632_s_at	N-myc downstream regulated gene 1 transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	NDRG1	1228	1604	1253	1234	2556	4199	4990	2905	1537	1873	5324	2876
202340_x_at	nuclear receptor subfamily 4, group A, member 1	NR4A1	56	112	130	74	2089	3897	4835	1356	720	721	5543	1029
206115_at	early growth response 3	EGR3	89	125	61	99	1134	2834	2776	1421	577	603	3720	1246
204622_x_at	nuclear receptor subfamily 4, group A, member 2	NR4A2	65	145	44	90	2999	5264	5600	2273	667	847	6917	799
208370_s_at	Down syndrome critical region gene 1	DSCR1	317	357	339	543	5927	7143	6541	5558	3129	1941	4974	2375
202388_at	regulator of G-protein signalling 2, 24kDa	RGS2	97	414	368	491	1161	2293	2154	1533	458	744	2076	693
209959_at	nuclear receptor subfamily 4, group A, member 3	NR4A3	22	50	26	37	935	1154	1216	265	59	64	2336	44
204472_at	GTP binding protein overexpressed in skeletal muscle	GEM	90	146	108	276	290	358	746	466	265	127	625	707
222162_s_at	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin	ADAMTS 1	562	316	510	956	2480	4939	7324	5268	757	910	7585	4226

Affymetrix probe set ID	Gene title	Gene symbol	Control				LTD_4				Thrombin			
			1	2	3	4	1	2	3	4	1	2	3	4
200632_s_at	N-myc downstream regulated gene 1 type 1 motif, 1	NDRG1	1228	1604	1253	1234	2556	4199	4990	2905	1537	1873	5324	2876
200797_s_at	myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	1963	1773	2281	1797	3412	7218	8922	3526	5688	7732	13834	7266
202672_s_at	activating transcription factor 3	ATF3	315	333	228	291	650	1748	1394	528	2162	1718	3222	2370
201694_s_at	early growth response 1	EGR1	424	616	461	703	804	2221	1584	1798	1032	1956	3234	2634
202859_x_at	Interleukin 8	IL8	125	190	157	361	816	2578	928	2736	4417	2411	2344	6428
204363_at	coagulation factor III (thromboplastin, tissue factor)	F3	14	34	22	21	354	387	377	438	194	207	97	227
205027_s_at	mitogen-activated protein kinase kinase kinase 8	MAP3K8	7	27	36	35	163	187	163	127	82	130	41	77
205290_s_at	bone morphogenetic protein 2	BMP2	2187	1579	893	1426	4325	5675	3806	3555	3470	2288	2296	2562
206211_at	selectin E (endothelial adhesion molecule 1)	SELE	21	30	6	107	201	164	66	246	378	92	34	122
209774_x_at	chemokine (C-X-C motif) ligand 2	CXCL2	104	176	102	676	818	3522	418	2408	882	453	513	1128
205960_at	pyruvate dehydrogenase kinase, isoenzyme 4	PDK4	360	509	487	328	999	1398	1532	572	521	572	730	293

Affymetrix probe set ID	Gene title	Gene symbol	Control				LTD_4				Thrombin			
			1	2	3	4	1	2	3	4	1	2	3	4
200632_s_at	N-myc downstream regulated gene 1	NDRG1	1228	1604	1253	1234	2556	4199	4990	2905	1537	1873	5324	2876
214446_at	elongation factor, RNA polymerase II, 2	ELL2	77	45	139	84	209	211	449	215	55	29	421	212

Table 8. Probe sets up-regulated (A) and down-regulated (B) >2-fold by LTD_4 at 1 h. Data were filtered according to the following criteria: detection *p*-value <0.065 at least in three of four samples in either the control or the stimulated group; signal value >150 at least in three of four samples in either the control or the stimulated group; fold change calculated from the signal means >2.0. Mean signals of four experiments are shown. n.a.: not annotated

A) Probe sets up-regulated >2-fold by LTD_4 at 1 h

Affymetrix probe set ID	Gene title	Gene symbol	Mean control	Mean LTD_4	Fold change LTD_4/control
204621_s_at	nuclear receptor subfamily 4, group A, member 2	NR4A2	**25**	**2682**	**104.5**
216248_s_at	nuclear receptor subfamily 4, group A, member 2	NR4A2	**61**	**4791**	**77.8**
204622_x_at	nuclear receptor subfamily 4, group A, member 2	NR4A2	**86**	**4034**	**46.9**
202340_x_at	nuclear receptor subfamily 4, group A, member 1	NR4A1	**93**	**3044**	**32.8**
209959_at	nuclear receptor subfamily 4, group A, member 3	NR4A3	**33**	**892**	**26.4**
206115_at	early growth response 3	EGR3	**93**	**2041**	**21.8**
204363_at	coagulation factor III (thromboplastin, tissue factor)	F3	**23**	**388**	**16.9**
208370_s_at	Down syndrome critical region gene 1	DSCR1	**388**	**6292**	**16.2**
204748_at	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	**281**	**4086**	**14.5**
205249_at	early growth response 2 (Krox-20 homolog, Drosophila)	EGR2	**91**	**861**	**9.4**
201531_at	zinc finger protein 36, C3H type, homolog (mouse)	ZFP36	**295**	**2763**	**9.4**
210764_s_at	cysteine-rich, angiogenic inducer, 61	CYR61	**906**	**8208**	**9.1**
207978_s_at	nuclear receptor subfamily 4, group A, member 3	NR4A3	**115**	**1002**	**8.7**
222162_s_at	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	ADAMTS1	**585**	**5002**	**8.5**

Affymetrix probe set ID	Gene title	Gene symbol	Mean control	Mean LTD_4	Fold change LTD_4/control
202859_x_at	Interleukin 8	IL8	**208**	**1764**	**8.5**
201044_x_at	dual specificity phosphatase 1	DUSP1	**47**	**373**	**7.9**
203821_at	heparin-binding EGF-like growth factor	HBEGF	**316**	**2363**	**7.5**
215253_s_at	Down syndrome critical region gene 1	DSCR1	**464**	**3390**	**7.3**
209774_x_at	chemokine (C-X-C motif) ligand 2	CXCL2	**264**	**1791**	**6.8**
201289_at	cysteine-rich, angiogenic inducer, 61	CYR61	**1135**	**7114**	**6.3**
205027_s_at	mitogen-activated protein kinase kinase kinase 8	MAP3K8	**26**	**159**	**6.1**
208078_s_at	SNF1-like kinase	SNF1LK	**408**	**2148**	**5.3**
202388_at	regulator of G-protein signalling 2, 24kDa	RGS2	**342**	**1785**	**5.2**
201041_s_at	dual specificity phosphatase 1	DUSP1	**1739**	**7852**	**4.5**
206211_at	selectin E (endothelial adhesion molecule 1)	SELE	**40**	**169**	**4.2**
38037_at	heparin-binding EGF-like growth factor	HBEGF	**218**	**880**	**4.0**
214438_at	H2.0-like homeo box 1 (Drosophila)	HLX1	**573**	**2303**	**4.0**
212614_at	AT rich interactive domain 5B (MRF1-like)	ARID5B	**467**	**1828**	**3.9**
221031_s_at	hypothetical protein DKFZp434F0318	DKFZP434 F0318	**669**	**2602**	**3.9**
221841_s_at	Kruppel-like factor 4 (gut)	KLF4	**424**	**1647**	**3.9**
202672_s_at	Activating transcription factor 3	ATF3	**291**	**1079**	**3.7**
209967_s_at	cAMP responsive element modulator	CREM	**517**	**1711**	**3.3**
206765_at	potassium inwardly-rectifying channel, subfamily J, member 2	KCNJ2	**806**	**2612**	**3.2**
211506_s_at	Interleukin 8	IL8	**173**	**550**	**3.2**
214446_at	Elongation factor, RNA polymerase II, 2	ELL2	**86**	**271**	**3.1**

Affymetrix probe set ID	Gene title	Gene symbol	Mean control	Mean LTD_4	Fold change LTD_4/control
218880_at	FOS-like antigen 2	FOSL2	**242**	**753**	**3.1**
202150_s_at	neural precursor cell expressed, developmentally down-regulated 9	NEDD9	**658**	**2034**	**3.1**
202149_at	neural precursor cell expressed, developmentally down-regulated 9	NEDD9	**697**	**2112**	**3.0**
204472_at	GTP binding protein overexpressed in skeletal muscle	GEM	**155**	**464**	**3.0**
200797_s_at	myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	**1953**	**5769**	**3.0**
209357_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	CITED2	**328**	**959**	**2.9**
201694_s_at	early growth response 1	EGR1	**551**	**1601**	**2.9**
205290_s_at	bone morphogenetic protein 2	BMP2	**1521**	**4340**	**2.9**
200632_s_at	N-myc downstream regulated gene 1	NDRG1	**1329**	**3662**	**2.8**
205960_at	pyruvate dehydrogenase kinase, isoenzyme 4	PDK4	**420**	**1125**	**2.7**
207980_s_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	CITED2	**345**	**916**	**2.7**
219371_s_at	Kruppel-like factor 2 (lung)	KLF2	**1114**	**2930**	**2.6**
214508_x_at	cAMP responsive element modulator	CREM	**552**	**1387**	**2.5**
207283_at	n.a.		**264**	**657**	**2.5**
36711_at	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	MAFF	**1768**	**4383**	**2.5**
200798_x_at	myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	**1624**	**4013**	**2.5**
220266_s_at	Kruppel-like factor 4 (gut)	KLF4	**202**	**500**	**2.5**

Affymetrix probe set ID	Gene title	Gene symbol	Mean control	Mean LTD_4	Fold change LTD_4/control
207630_s_at	cAMP responsive element modulator	CREM	**992**	**2427**	**2.4**
213260_at	Forkhead box C1	FOXC1	**1314**	**3204**	**2.4**
212558_at	sprouty homolog 1, antagonist of FGF signaling (Drosophila)	SPRY1	**599**	**1440**	**2.4**
200796_s_at	myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	**424**	**1018**	**2.4**
205289_at	bone morphogenetic protein 2	BMP2	**1287**	**3089**	**2.4**
204011_at	sprouty homolog 2 (Drosophila)	SPRY2	**510**	**1189**	**2.3**
209074_s_at	TU3A protein	TU3A	**127**	**293**	**2.3**
218541_s_at	chromosome 8 open reading frame 4	C8orf4	**150**	**345**	**2.3**
204135_at	downregulated in ovarian cancer 1	DOC1	**551**	**1259**	**2.3**
204285_s_at	phorbol-12-myristate-13-acetate-induced protein 1	PMAIP1	**196**	**430**	**2.2**
208960_s_at	Kruppel-like factor 6	KLF6	**879**	**1925**	**2.2**
218886_at	PAK1 interacting protein 1	PAK1IP1	**98**	**215**	**2.2**
208415_x_at	inhibitor of growth family, member 1	ING1	**228**	**498**	**2.2**
212665_at	TCDD-inducible poly(ADP-ribose) polymerase	TIPARP	**531**	**1154**	**2.2**
218881_s_at	FOS-like antigen 2	FOSL2	**216**	**461**	**2.1**
201170_s_at	basic helix-loop-helix domain containing, class B, 2	BHLHB2	**1039**	**2212**	**2.1**
209457_at	dual specificity phosphatase 5	DUSP5	**1213**	**2533**	**2.1**
205409_at	FOS-like antigen 2	FOSL2	**145**	**296**	**2.0**
203868_s_at	vascular cell adhesion molecule 1	VCAM1	**163**	**331**	**2.0**

B) Probe sets down-regulated >2-fold by LTD_4 at 1 h

Affymetrix probe set ID	Gene title	Gene symbol	Mean control	Mean LTD_4	Fold change −control/LTD_4
201008_s_at	thioredoxin interacting protein	TXNIP	**2090**	**958**	**-2.2**
201009_s_at	thioredoxin interacting protein	TXNIP	**2729**	**1260**	**-2.2**
215521_at	polyhomeotic like 3 (Drosophila)	PHC3	**144**	**71**	**-2.0**
208937_s_at	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	ID1	**2806**	**1395**	**-2.0**

Table 9. Probe sets up-regulated (A) and down-regulated (B) >2-fold by thrombin at 1 h. Data were filtered according to the following criteria: detection *p*-value <0.065 at least in three of four samples in either the control or the stimulated group; signal value >150 at least in three of four samples in either the control or the stimulated group; fold change calculated from the signal means >2.0. Mean signals of four experiments are shown.

A) Probe sets up-regulated >2-fold by thrombin at 1 h

Affymetrix probe set ID	Gene title	Gene symbol	Mean control	Mean thrombin	Fold change thrombin/control
202768_at	FBJ murine osteosarcoma viral oncogene homolog B	FOSB	**25**	**3250**	**126.4**
204621_s_at	nuclear receptor subfamily 4, group A, member 2	NR4A2	**25**	**1459**	**56.8**
216248_s_at	nuclear receptor subfamily 4, group A, member 2	NR4A2	**61**	**2693**	**43.8**
204622_x_at	nuclear receptor subfamily 4, group A, member 2	NR4A2	**86**	**2307**	**26.8**
202340_x_at	nuclear receptor subfamily 4, group A, member 1	NR4A1	**93**	**2003**	**21.6**
202859_x_at	interleukin 8	IL8	**208**	**3900**	**18.7**
206115_at	early growth response 3	EGR3	**93**	**1536**	**16.4**
210764_s_at	cysteine-rich, angiogenic inducer, 61	CYR61	**906**	**13920**	**15.4**
203821_at	heparin-binding EGF-like growth factor	HBEGF	**316**	**3248**	**10.3**
201289_at	cysteine-rich, angiogenic inducer, 61	CYR61	**1135**	**10610**	**9.3**
202672_s_at	Activating transcription factor 3	ATF3	**291**	**2368**	**8.1**
208370_s_at	Down syndrome critical region gene 1	DSCR1	**388**	**3104**	**8.0**
204748_at	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	**281**	**2128**	**7.6**

Affymetrix probe set ID	Gene title	Gene symbol	Mean control	Mean thrombin	Fold change thrombin/control
201531_at	zinc finger protein 36, C3H type, homolog (mouse)	ZFP36	**295**	**2214**	**7.5**
205249_at	early growth response 2 (Krox-20 homolog, Drosophila)	EGR2	**91**	**643**	**7.0**
211506_s_at	interleukin 8	IL8	**173**	**1204**	**6.9**
207978_s_at	nuclear receptor subfamily 4, group A, member 3	NR4A3	**115**	**793**	**6.9**
38037_at	heparin-binding EGF-like growth factor	HBEGF	**218**	**1407**	**6.4**
221841_s_at	Kruppel-like factor 4 (gut)	KLF4	**424**	**2591**	**6.1**
222162_s_at	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	ADAMTS1	**585**	**3369**	**5.8**
208078_s_at	SNF1-like kinase	SNF1LK	**408**	**2128**	**5.2**
204135_at	downregulated in ovarian cancer 1	DOC1	**551**	**2782**	**5.0**
204222_s_at	GLI pathogenesis-related 1 (glioma)	GLIPR1	**51**	**255**	**5.0**
215198_s_at	caldesmon 1	CALD1	**145**	**656**	**4.5**
220266_s_at	Kruppel-like factor 4 (gut)	KLF4	**202**	**907**	**4.5**
200797_s_at	myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	**1953**	**8630**	**4.4**
215199_at	caldesmon 1	CALD1	**117**	**517**	**4.4**
201041_s_at	dual specificity phosphatase 1	DUSP1	**1739**	**7313**	**4.2**
201466_s_at	v-jun sarcoma virus 17 oncogene homolog (avian)	JUN	**283**	**1165**	**4.1**
201694_s_at	early growth response 1	EGR1	**551**	**2213**	**4.0**
202270_at	guanylate binding protein 1, interferon-inducible, 67kDa	GBP1	**64**	**251**	**3.9**

Affymetrix probe set ID	Gene title	Gene symbol	Mean control	Mean thrombin	Fold change thrombin/control
212614_at	AT rich interactive domain 5B (MRF1-like)	ARID5B	**467**	**1802**	**3.9**
202150_s_at	neural precursor cell expressed, developmentally down-regulated 9	NEDD9	**658**	**2441**	**3.7**
218541_s_at	chromosome 8 open reading frame 4	C8orf4	**150**	**549**	**3.7**
200796_s_at	myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	**424**	**1502**	**3.5**
200798_x_at	myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	**1624**	**5724**	**3.5**
208960_s_at	Kruppel-like factor 6	KLF6	**879**	**3000**	**3.4**
215253_s_at	Down syndrome critical region gene 1	DSCR1	**464**	**1535**	**3.3**
204470_at	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	CXCL1	**135**	**446**	**3.3**
209101_at	connective tissue growth factor	CTGF	**5531**	**18122**	**3.3**
202149_at	neural precursor cell expressed, developmentally down-regulated 9	NEDD9	**697**	**2280**	**3.3**
208119_s_at	zinc finger protein 505	ZNF505	**129**	**422**	**3.3**
201739_at	serum/glucocorticoid regulated kinase	SGK	**1045**	**3371**	**3.2**
218880_at	FOS-like antigen 2	FOSL2	**242**	**779**	**3.2**
221031_s_at	hypothetical protein DKFZp434F0318	DKFZP434F0318	**669**	**2137**	**3.2**
208961_s_at	Kruppel-like factor 6	KLF6	**1540**	**4841**	**3.1**
202269_x_at	guanylate binding protein 1, interferon-inducible, 67kDa	GBP1	**187**	**585**	**3.1**

Affymetrix probe set ID	Gene title	Gene symbol	Mean control	Mean thrombin	Fold change thrombin/control
214438_at	H2.0-like homeo box 1 (Drosophila)	HLX1	**573**	**1718**	**3.0**
202388_at	regulator of G-protein signalling 2, 24kDa	RGS2	**342**	**992**	**2.9**
209357_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	CITED2	**328**	**932**	**2.8**
201465_s_at	v-jun sarcoma virus 17 oncogene homolog (avian)	JUN	**185**	**525**	**2.8**
209774_x_at	chemokine (C-X-C motif) ligand 2	CXCL2	**264**	**743**	**2.8**
204472_at	GTP binding protein overexpressed in skeletal muscle	GEM	**155**	**431**	**2.8**
207980_s_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	CITED2	**345**	**958**	**2.8**
214085_x_at	GLI pathogenesis-related 1 (glioma)	GLIPR1	**302**	**825**	**2.7**
204011_at	sprouty homolog 2 (Drosophila)	SPRY2	**510**	**1342**	**2.6**
219371_s_at	Kruppel-like factor 2 (lung)	KLF2	**1114**	**2756**	**2.5**
202067_s_at	low density lipoprotein receptor (familial hypercholesterolemia)	LDLR	**192**	**475**	**2.5**
201464_x_at	v-jun sarcoma virus 17 oncogene homolog (avian)	JUN	**1581**	**3866**	**2.4**
218881_s_at	FOS-like antigen 2	FOSL2	**216**	**519**	**2.4**
202241_at	tribbles homolog 1 (Drosophila)	TRIB1	**712**	**1712**	**2.4**
206765_at	potassium inwardly-rectifying channel, subfamily J, member 2	KCNJ2	**806**	**1935**	**2.4**
203751_x_at	jun D proto-oncogene	JUND	**381**	**887**	**2.3**

Affymetrix probe set ID	Gene title	Gene symbol	Mean control	Mean thrombin	Fold change thrombin/control
201693_s_at	early growth response 1	EGR1	**268**	**623**	**2.3**
212418_at	E74-like factor 1 (ets domain transcription factor)	ELF1	**543**	**1234**	**2.3**
218995_s_at	endothelin 1	EDN1	**1492**	**3370**	**2.3**
200632_s_at	N-myc downstream regulated gene 1	NDRG1	**1329**	**2902**	**2.2**
220990_s_at	likely ortholog of rat vacuole membrane protein 1	VMP1	**1223**	**2646**	**2.2**
202014_at	protein phosphatase 1, regulatory (inhibitor) subunit 15A	PPP1R15A	**413**	**880**	**2.1**
202068_s_at	low density lipoprotein receptor (familial hypercholesterolemia)	LDLR	**1672**	**3509**	**2.1**
204221_x_at	GLI pathogenesis-related 1 (glioma)	GLIPR1	**138**	**280**	**2.0**
203140_at	B-cell CLL/lymphoma 6 (zinc finger protein 51)	BCL6	**381**	**769**	**2.0**
215206_at	Exostoses (multiple) 1	EXT1	**230**	**463**	**2.0**

B) Probe sets down-regulated >2-fold by thrombin at 1 h

Affymetrix probe set ID	Gene title	Gene symbol	Mean control	Mean thrombin	Fold change −control/thrombin
201367_s_at	zinc finger protein 36, C3H type-like 2	ZFP36L2	**876**	**208**	**-4.2**
202887_s_at	DNA-damage-inducible transcript 4	DDIT4	**1963**	**523**	**-3.7**
201368_at	zinc finger protein 36, C3H type-like 2	ZFP36L2	**4562**	**1399**	**-3.3**

Affymetrix probe set ID	Gene title	Gene symbol	Mean control	Mean thrombin	Fold change −control/thrombin
208937_s_at	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	ID1	**2806**	**870**	**-3.2**
201369_s_at	zinc finger protein 36, C3H type-like 2	ZFP36L2	**666**	**233**	**-2.9**
209905_at	homeo box A9	HOXA9	**438**	**166**	**-2.6**
209602_s_at	GATA binding protein 3	GATA3	**195**	**76**	**-2.6**
203395_s_at	hairy and enhancer of split 1, (Drosophila)	HES1	**1098**	**431**	**-2.5**
201010_s_at	thioredoxin interacting protein	TXNIP	**3484**	**1397**	**-2.5**
203394_s_at	hairy and enhancer of split 1, (Drosophila)	HES1	**1347**	**544**	**-2.5**
201008_s_at	thioredoxin interacting protein	TXNIP	**2090**	**866**	**-2.4**
201009_s_at	thioredoxin interacting protein	TXNIP	**2729**	**1138**	**-2.4**
207826_s_at	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	ID3	**2898**	**1294**	**-2.2**
205020_s_at	ADP-ribosylation factor-like 4A	ARL4A	**2031**	**956**	**-2.1**
207474_at	SNF related kinase	SNRK	**187**	**90**	**-2.1**

Table 10. Probe sets up-regulated (A) and down-regulated (B) >2-fold by LTD_4 at 6 h. Data were filtered according to the following criteria: detection *p*-value <0.065 and signal value >150 in either the control or the stimulated group; fold change >2.0. Results of one experiment are shown.

A) Probe sets up-regulated >2-fold by LTD_4 at 6 h

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4	Fold change LTD_4/Control
206942_s_at	pro-melanin-concentrating hormone	PMCH	**16**	**1227**	**77.7**
220351_at	chemokine (C-C motif) receptor-like 1	CCRL1	**36**	**245**	**6.7**
205902_at	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3	KCNN3	**118**	**662**	**5.6**
207016_s_at	aldehyde dehydrogenase 1 family, member A2	ALDH1A2	**537**	**2263**	**4.2**
215012_at	zinc finger protein 451	ZNF451	**77**	**281**	**3.7**
204818_at	hydroxysteroid (17-beta) dehydrogenase 2	HSD17B2	**83**	**302**	**3.6**
212927_at	SMC5 structural maintenance of chromosomes 5-like 1 (yeast)	SMC5L1	**53**	**174**	**3.3**
210946_at	phosphatidic acid phosphatase type 2A	PPAP2A	**259**	**821**	**3.2**
202388_at	regulator of G-protein signalling 2, 24kDa	RGS2	**147**	**430**	**2.9**
212730_at	desmuslin	DMN	**382**	**1107**	**2.9**
218935_at	EH-domain containing 3	EHD3	**507**	**1412**	**2.8**
208370_s_at	Down syndrome critical region gene 1	DSCR1	**307**	**818**	**2.7**
209147_s_at	phosphatidic acid phosphatase type 2A	PPAP2A	**466**	**1235**	**2.6**
214987_at	GRB2-associated binding protein 1	GAB1	**67**	**176**	**2.6**
202291_s_at	matrix Gla protein	MGP	**2960**	**7428**	**2.5**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4	Fold change LTD_4/Control
203282_at	glucan (1,4-alpha-), branching enzyme 1 (glycogen branching enzyme, Andersen disease, glycogen storage disease type IV)	GBE1	**825**	**2051**	**2.5**
206103_at	ras-related C3 botulinum toxin substrate 3 (rho family, small GTP binding protein Rac3)	RAC3	**67**	**164**	**2.5**
201641_at	bone marrow stromal cell antigen 2	BST2	**63**	**153**	**2.4**
215930_s_at	CTAGE family, member 5	CTAGE5	**67**	**159**	**2.4**
203023_at	hypothetical protein HSPC111	HSPC111	**97**	**227**	**2.3**
220037_s_at	extracellular link domain containing 1	XLKD1	**394**	**909**	**2.3**
207015_s_at	aldehyde dehydrogenase 1 family, member A2	ALDH1A2	**178**	**404**	**2.3**
206167_s_at	Rho GTPase activating protein 6	ARHGAP6	**97**	**219**	**2.3**
34206_at	centaurin, delta 2	CENTD2	**233**	**522**	**2.2**
211993_at	WNK lysine deficient protein kinase 1	WNK1	**86**	**190**	**2.2**
213388_at	phosphodiesterase 4D interacting protein (myomegalin)	PDE4DIP	**89**	**197**	**2.2**
219534_x_at	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	CDKN1C	**115**	**249**	**2.2**
220241_at	transmembrane and coiled-coil domains 3	TMCO3	**84**	**181**	**2.2**
201925_s_at	decay accelerating factor for complement (CD55, Cromer blood group system)	DAF	**1006**	**2167**	**2.2**
201860_s_at	plasminogen activator, tissue	PLAT	**238**	**512**	**2.2**
205903_s_at	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3	KCNN3	**151**	**325**	**2.1**
213853_at	zinc finger, CSL-type containing 3	ZCSL3	**229**	**491**	**2.1**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4	Fold change LTD_4/Control
210723_x_at	hypothetical protein MGC4771	MGC4771	**75**	**161**	**2.1**
210195_s_at	pregnancy specific beta-1-glycoprotein 1	PSG1	**78**	**165**	**2.1**
213629_x_at	metallothionein 1F (functional)	MT1F	**164**	**344**	**2.1**
204596_s_at	stanniocalcin 1	STC1	**1297**	**2707**	**2.1**
209277_at	Tissue factor pathway inhibitor 2	TFPI2	**818**	**1694**	**2.1**
214844_s_at	docking protein 5	DOK5	**381**	**786**	**2.1**
212306_at	cytoplasmic linker associated protein 2	CLASP2	**479**	**985**	**2.1**
207936_x_at	ret finger protein-like 3	RFPL3	**170**	**346**	**2.0**
215054_at	erythropoietin receptor	EPOR	**244**	**496**	**2.0**
219059_s_at	extracellular link domain containing 1	XLKD1	**581**	**1164**	**2.0**

B) Probe sets down-regulated >2-fold by LTD_4 at 6 h

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4	Fold change –Control/LTD_4
48030_i_at	chromosome 5 open reading frame 4	C5orf4	**2274**	**273**	**-8.3**
213033_s_at	Nuclear factor I/B	NFIB	**874**	**224**	**-3.9**
204162_at	kinetochore associated 2	KNTC2	**160**	**53**	**-3.0**
204573_at	carnitine O-octanoyltransferase	CROT	**334**	**113**	**-2.9**
219336_s_at	activating signal cointegrator 1 complex subunit 1	ASCC1	**309**	**110**	**-2.8**
214829_at	aminoadipate-semialdehyde synthase	AASS	**193**	**69**	**-2.8**
218179_s_at	FLJ12716 protein	FLJ12716	**274**	**100**	**-2.7**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4	Fold change –Control/LTD_4
207891_s_at	three prime repair exonuclease 2	TREX2	**180**	**66**	**-2.7**
220813_at	cysteinyl leukotriene receptor 2	CYSLTR2	**624**	**231**	**-2.7**
204833_at	ATG12 autophagy related 12 homolog (S. cerevisiae)	ATG12	**162**	**61**	**-2.7**
204535_s_at	RE1-silencing transcription factor	REST	**226**	**96**	**-2.4**
222278_at	hypothetical LOC389393	LOC389393	**309**	**133**	**-2.3**
213264_at	Poly(rC) binding protein 2	PCBP2	**288**	**127**	**-2.3**
219805_at	hypothetical protein FLJ22965	FLJ22965	**152**	**69**	**-2.2**
209821_at	chromosome 9 open reading frame 26 (NF-HEV)	C9orf26	**767**	**352**	**-2.2**
214787_at	c-myc promoter binding protein	MYCPBP	**234**	**108**	**-2.2**
207361_at	HMG-box transcription factor 1	HBP1	**163**	**76**	**-2.1**
47560_at	latrophilin 1	LPHN1	**687**	**324**	**-2.1**
219383_at	hypothetical protein FLJ14213	FLJ14213	**171**	**81**	**-2.1**
204098_at	RNA binding motif protein, X-linked 2	RBMX2	**421**	**202**	**-2.1**
211241_at	annexin A2 pseudogene 3	ANXA2P3	**389**	**187**	**-2.1**
220732_at	DEP domain containing 2	DEPDC2	**254**	**123**	**-2.1**
218643_s_at	postsynaptic protein CRIPT	CRIPT	**155**	**75**	**-2.1**
207542_s_at	aquaporin 1 (channel-forming integral protein, 28kDa)	AQP1	**4729**	**2345**	**-2.0**

Table 11. Probe sets up-regulated (A) or down-regulated (B) >2-fold by LTD_4 at 24 h. Data were filtered according to the following criteria: detection *p*-value <0.065 and signal value >150 in either the control or the stimulated group; fold change >2.0. Results of one experiment are shown. n.a.: not annotated

A) Probe sets up-regulated >2-fold by LTD_4 at 24 h

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4	Fold change LTD_4/Control
206942_s_at	pro-melanin-concentrating hormone	PMCH	**100**	**650**	**6.5**
214617_at	perforin 1 (pore forming protein)	PRF1	**27**	**153**	**5.7**
210615_at	neuropilin 1	NRP1	**44**	**184**	**4.2**
204197_s_at	runt-related transcription factor 3	RUNX3	**47**	**193**	**4.1**
207191_s_at	immunoglobulin superfamily containing leucine-rich repeat	ISLR	**53**	**210**	**3.9**
210809_s_at	periostin, osteoblast specific factor	POSTN	**114**	**427**	**3.7**
204340_at	chromosome X open reading frame 12	CXorf12	**74**	**264**	**3.6**
202921_s_at	ankyrin 2, neuronal	ANK2	**76**	**239**	**3.2**
208266_at	chromosome 8 open reading frame 17	C8orf17	**50**	**157**	**3.1**
202291_s_at	matrix Gla protein	MGP	**3100**	**9590**	**3.1**
214880_x_at	caldesmon 1	CALD1	**177**	**543**	**3.1**
215057_at	CDNA FLJ44451 fis, clone UTERU2023039		**58**	**170**	**2.9**
218444_at	asparagine-linked glycosylation 12 homolog (yeast, alpha-1,6-mannosyltransferase)	ALG12	**75**	**215**	**2.9**
219503_s_at	transmembrane protein 40	TMEM40	**84**	**233**	**2.8**
204116_at	interleukin 2 receptor, gamma (severe combined immunodeficiency)	IL2RG	**136**	**373**	**2.7**
213622_at	collagen, type IX, alpha 2	COL9A2	**62**	**168**	**2.7**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4	Fold change LTD_4/Control
214529_at	thyroid stimulating hormone, beta	TSHB	**107**	**280**	**2.6**
201860_s_at	plasminogen activator, tissue	PLAT	**153**	**395**	**2.6**
208138_at	gastrin	GAST	**92**	**236**	**2.6**
206734_at	jerky homolog-like (mouse)	JRKL	**155**	**394**	**2.5**
208246_x_at	Thymidine kinase 2, mitochondrial	TK2	**206**	**522**	**2.5**
208588_at	apoptosis inhibitor	FKSG2	**87**	**220**	**2.5**
60794_f_at	zinc finger protein 587	ZNF587	**66**	**162**	**2.4**
202500_at	DnaJ (Hsp40) homolog, subfamily B, member 2	DNAJB2	**90**	**217**	**2.4**
213605_s_at	Glucuronidase, beta-like 1	C6orf216	**102**	**247**	**2.4**
206167_s_at	Rho GTPase activating protein 6	ARHGAP6	**80**	**193**	**2.4**
201242_s_at	ATPase, Na+/K+ transporting, beta 1 polypeptide	ATP1B1	**130**	**302**	**2.3**
202774_s_at	splicing factor, arginine/serine-rich 8 (suppressor-of-white-apricot homolog, Drosophila)	SFRS8	**362**	**801**	**2.2**
203213_at	Cell division cycle 2, G1 to S and G2 to M	CDC2	**282**	**623**	**2.2**
213873_at	n.a.		**80**	**176**	**2.2**
202995_s_at	fibulin 1	FBLN1	**80**	**174**	**2.2**
220558_x_at	tetraspanin 32	TSPAN32	**115**	**250**	**2.2**
205939_at	cytochrome P450, family 3, subfamily A, polypeptide 7	CYP3A7	**91**	**199**	**2.2**
214033_at	ATP-binding cassette, sub-family C (CFTR/MRP), member 6	ABCC6	**70**	**152**	**2.2**
208617_s_at	protein tyrosine phosphatase type IVA, member 2	PTP4A2	**1047**	**2273**	**2.2**
207591_s_at	AT rich interactive domain 1A (SWI- like)	ARID1A	**77**	**165**	**2.1**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4	Fold change LTD_4/Control
214057_at	Myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	**92**	**195**	**2.1**
204081_at	neurogranin (protein kinase C substrate, RC3)	NRGN	**238**	**503**	**2.1**
211892_s_at	prostaglandin I2 (prostacyclin) synthase	PTGIS	**85**	**178**	**2.1**
211685_s_at	neurocalcin delta	NCALD	**79**	**167**	**2.1**
218712_at	chromosome 1 open reading frame 109	C1orf109	**78**	**163**	**2.1**
207152_at	neurotrophic tyrosine kinase, receptor, type 2	NTRK2	**169**	**354**	**2.1**
222155_s_at	G protein-coupled receptor 172A	GPR172A	**162**	**338**	**2.1**
214379_at	BMX non-receptor tyrosine kinase	BMX	**82**	**171**	**2.1**
213654_at	TAF5-like RNA polymerase II, p300/CBP-associated factor (PCAF)-associated factor, 65kDa	TAF5L	**322**	**668**	**2.1**
219102_at	reticulocalbin 3, EF-hand calcium binding domain	RCN3	**142**	**295**	**2.1**
213976_at	CDKN1A interacting zinc finger protein 1	CIZ1	**85**	**175**	**2.1**
208370_s_at	Down syndrome critical region gene 1	DSCR1	**358**	**740**	**2.1**
210564_x_at	CASP8 and FADD-like apoptosis regulator	CFLAR	**361**	**747**	**2.1**
209176_at	SEC23 interacting protein	SEC23IP	**142**	**294**	**2.1**
209642_at	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	BUB1	**101**	**208**	**2.1**
204887_s_at	polo-like kinase 4 (Drosophila)	PLK4	**123**	**252**	**2.1**
209172_s_at	centromere protein F, 350/400ka (mitosin)	CENPF	**95**	**193**	**2.0**
210484_s_at	tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	TNFRSF10C	**158**	**322**	**2.0**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4	Fold change LTD_4/Control
216490_x_at	similar to dJ408B20.3 (novel protein similar to 60S acidic ribosomal protein P2 (RPLP2))	LOC442175	**76**	**155**	**2.0**
214519_s_at	relaxin 2	RLN2	**78**	**158**	**2.0**
218746_at	TAP binding protein-like	TAPBPL	**98**	**198**	**2.0**
213215_at	MRNA full length insert cDNA clone EUROIMAGE 42138		**192**	**387**	**2.0**
204918_s_at	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3	MLLT3	**82**	**164**	**2.0**

B) Probe sets down-regulated >2-fold by LTD_4 at 24 h

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4	Fold change –Control/LTD_4
221586_s_at	E2F transcription factor 5, p130-binding	E2F5	**279**	**31**	**-8.9**
217588_at	cation channel, sperm associated 2	CATSPER2	**174**	**31**	**-5.7**
219077_s_at	WW domain containing oxidoreductase	WWOX	**312**	**57**	**-5.5**
219661_at	RAN binding protein 17	RANBP17	**255**	**89**	**-2.9**
216514_at	n.a.		**191**	**67**	**-2.8**
201396_s_at	small glutamine-rich tetratricopeptide repeat (TPR)-containing, alpha	SGTA	**182**	**67**	**-2.7**
204349_at	cofactor required for Sp1 transcriptional activation, subunit 9, 33kDa	CRSP9	**152**	**56**	**-2.7**
214314_s_at	eukaryotic translation initiation factor 5B	EIF5B	**158**	**60**	**-2.6**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4	Fold change –Control/LTD_4
216882_s_at	nebulette	NEBL	**210**	**83**	**-2.5**
207831_x_at	deoxyhypusine synthase	DHPS	**177**	**75**	**-2.4**
213074_at	Interleukin-1 receptor-associated kinase 1 binding protein 1	IRAK1BP1	**675**	**288**	**-2.3**
215825_at	Clone 24487 mRNA sequence		**236**	**103**	**-2.3**
212475_at	KIAA0241 protein	KIAA0241	**254**	**112**	**-2.3**
203098_at	chromodomain protein, Y-like	CDYL	**495**	**220**	**-2.3**
202432_at	protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform (calcineurin A beta)	PPP3CB	**602**	**268**	**-2.2**
218569_s_at	kelch repeat and BTB (POZ) domain containing 4	KBTBD4	**211**	**103**	**-2.0**
205887_x_at	mutS homolog 3 (E. coli)	MSH3	**182**	**89**	**-2.0**
212060_at	U2-associated SR140 protein	SR140	**269**	**132**	**-2.0**
209469_at	glycoprotein M6A	GPM6A	**161**	**80**	**-2.0**

Table 12. Probe sets up-regulated (A) or down-regulated (B) >2-fold by thrombin at 6 h. Data were filtered according to the following criteria: detection *p*-value <0.065 and signal value >150 in either the control or the stimulated group; fold change >2.0. Results of one experiment are shown. n.a.: not annotated

A) Probe sets up-regulated >2-fold by thrombin at 6 h

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change Thrombin/Control
214446_at	elongation factor, RNA polymerase II, 2	ELL2	**21**	**177**	**8.6**
204222_s_at	GLI pathogenesis-related 1 (glioma)	GLIPR1	**47**	**356**	**7.5**
206211_at	selectin E (endothelial adhesion molecule 1)	SELE	**47**	**339**	**7.2**
202859_x_at	interleukin 8	IL8	**164**	**1119**	**6.8**
203836_s_at	mitogen-activated protein kinase kinase kinase 5	MAP3K5	**46**	**245**	**5.4**
203868_s_at	vascular cell adhesion molecule 1	VCAM1	**80**	**333**	**4.2**
210764_s_at	cysteine-rich, angiogenic inducer, 61	CYR61	**1119**	**4382**	**3.9**
204748_at	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	**84**	**324**	**3.9**
202627_s_at	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	SERPINE1	**3515**	**13468**	**3.8**
201242_s_at	ATPase, Na+/K+ transporting, beta 1 polypeptide	ATP1B1	**245**	**917**	**3.7**
202638_s_at	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	ICAM1	**251**	**913**	**3.6**
211506_s_at	interleukin 8	IL8	**127**	**406**	**3.2**
203666_at	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	CXCL12	**248**	**791**	**3.2**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change Thrombin/Control
202637_s_at	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	ICAM1	**355**	**1114**	**3.1**
210939_s_at	glutamate receptor, metabotropic 1	GRM1	**60**	**186**	**3.1**
201243_s_at	ATPase, Na+/K+ transporting, beta 1 polypeptide	ATP1B1	**325**	**972**	**3.0**
214844_s_at	docking protein 5	DOK5	**381**	**1134**	**3.0**
221085_at	tumor necrosis factor (ligand) superfamily, member 15	TNFSF15	**97**	**282**	**2.9**
219973_at	arylsulfatase J	ARSJ	**82**	**236**	**2.9**
220116_at	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	KCNN2	**141**	**399**	**2.8**
213338_at	Ras-induced senescence 1	RIS1	**82**	**231**	**2.8**
201289_at	cysteine-rich, angiogenic inducer, 61	CYR61	**1787**	**4906**	**2.7**
213853_at	zinc finger, CSL-type containing 3	ZCSL3	**229**	**623**	**2.7**
205599_at	TNF receptor-associated factor 1	TRAF1	**84**	**225**	**2.7**
209373_at	mal, T-cell differentiation protein-like	MALL	**699**	**1859**	**2.7**
215495_s_at	sterile alpha motif domain containing 4	SAMD4	**67**	**178**	**2.7**
202628_s_at	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	SERPINE1	**3762**	**9813**	**2.6**
205680_at	matrix metallopeptidase 10 (stromelysin 2)	MMP10	**359**	**933**	**2.6**
205463_s_at	platelet-derived growth factor alpha polypeptide	PDGFA	**311**	**806**	**2.6**
210593_at	spermidine/spermine N1-acetyltransferase	SAT	**63**	**162**	**2.6**
216598_s_at	chemokine (C-C motif) ligand 2	CCL2	**1267**	**3139**	**2.5**
207665_at	ADAM metallopeptidase domain 21	ADAM21	**95**	**236**	**2.5**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change Thrombin/Control
206157_at	pentraxin-related gene, rapidly induced by IL-1 beta	PTX3	**764**	**1877**	**2.5**
213093_at	protein kinase C, alpha	PRKCA	**102**	**248**	**2.4**
215223_s_at	superoxide dismutase 2, mitochondrial	SOD2	**317**	**755**	**2.4**
218498_s_at	ERO1-like (S. cerevisiae)	ERO1L	**166**	**391**	**2.3**
213629_x_at	metallothionein 1F (functional)	MT1F	**164**	**383**	**2.3**
216256_at	glutamate receptor, metabotropic 8	GRM8	**88**	**204**	**2.3**
208394_x_at	endothelial cell-specific molecule 1	ESM1	**366**	**850**	**2.3**
205132_at	actin, alpha, cardiac muscle	ACTC	**119**	**276**	**2.3**
211124_s_at	KIT ligand	KITLG	**204**	**470**	**2.3**
217477_at	phosphatidylinositol-4-phosphate 5-kinase, type I, beta	PIP5K1B	**68**	**156**	**2.3**
209598_at	paraneoplastic antigen MA2	PNMA2	**212**	**488**	**2.3**
209372_x_at	tubulin, beta 2	TUBB2	**118**	**272**	**2.3**
218330_s_at	neuron navigator 2	NAV2	**286**	**656**	**2.3**
218368_s_at	tumor necrosis factor receptor superfamily, member 12A	TNFRSF12A	**433**	**990**	**2.3**
206116_s_at	tropomyosin 1 (alpha)	TPM1	**692**	**1580**	**2.3**
203023_at	hypothetical protein HSPC111	HSPC111	**97**	**221**	**2.3**
203999_at	synaptotagmin I	SYT1	**170**	**386**	**2.3**
207526_s_at	interleukin 1 receptor-like 1	IL1RL1	**129**	**289**	**2.2**
207536_s_at	tumor necrosis factor receptor superfamily, member 9	TNFRSF9	**80**	**179**	**2.2**
218414_s_at	nudE nuclear distribution gene E homolog 1 (A. nidulans)	NDE1	**132**	**295**	**2.2**
201246_s_at	OTU domain, ubiquitin aldehyde binding 1	OTUB1	**124**	**277**	**2.2**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change Thrombin/Control
216733_s_at	glycine amidinotransferase (L-arginine:glycine amidinotransferase)	GATM	**68**	**151**	**2.2**
216855_s_at	heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	HNRPU	**236**	**519**	**2.2**
214085_x_at	GLI pathogenesis-related 1 (glioma)	GLIPR1	**161**	**353**	**2.2**
204475_at	matrix metallopeptidase 1 (interstitial collagenase)	MMP1	**374**	**811**	**2.2**
204135_at	downregulated in ovarian cancer 1	DOC1	**651**	**1411**	**2.2**
214173_x_at	chromosome 19 open reading frame 2	C19orf2	**390**	**840**	**2.2**
208960_s_at	Kruppel-like factor 6	KLF6	**1048**	**2251**	**2.1**
210524_x_at	n.a.		**144**	**307**	**2.1**
205305_at	fibrinogen-like 1	FGL1	**115**	**245**	**2.1**
206096_at	zinc finger protein 35 (clone HF.10)	ZNF35	**163**	**346**	**2.1**
205572_at	angiopoietin 2	ANGPT2	**1803**	**3822**	**2.1**
204200_s_at	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	PDGFB	**366**	**772**	**2.1**
205232_s_at	platelet-activating factor acetylhydrolase 2, 40kDa	PAFAH2	**98**	**206**	**2.1**
201995_at	exostoses (multiple) 1	EXT1	**690**	**1452**	**2.1**
214482_at	zinc finger and BTB domain containing 25	ZBTB25	**81**	**170**	**2.1**
201012_at	annexin A1	ANXA1	**3778**	**7902**	**2.1**
201058_s_at	myosin, light polypeptide 9, regulatory	MYL9	**254**	**530**	**2.1**
220542_s_at	palate, lung and nasal epithelium carcinoma associated	PLUNC	**128**	**266**	**2.1**
210723_x_at	hypothetical protein MGC4771	MGC4771	**75**	**156**	**2.1**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change Thrombin/Control
221443_x_at	prolactin releasing hormone	PRLH	**101**	**208**	**2.1**
209305_s_at	growth arrest and DNA-damage-inducible, beta	GADD45B	**124**	**254**	**2.0**
209508_x_at	CASP8 and FADD-like apoptosis regulator	CFLAR	**378**	**770**	**2.0**
211456_x_at	Similar to 60S ribosomal protein L35	RPL35	**561**	**1140**	**2.0**
823_at	chemokine (C-X3-C motif) ligand 1	CX3CL1	**147**	**300**	**2.0**
217579_x_at	ADP-ribosylation factor-like 6 interacting protein 2	ARL6IP2	**212**	**430**	**2.0**

B) Probe sets down-regulated >2-fold by thrombin at 6 h

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change –Control/Thrombin
214613_at	G protein-coupled receptor 3	GPR3	**161**	**30**	**-5.5**
87100_at	Abhydrolase domain containing 2	ABHD2	**203**	**52**	**-3.9**
222278_at	hypothetical LOC389393	LOC389393	**309**	**81**	**-3.8**
205020_s_at	ADP-ribosylation factor-like 4	ARL4	**2083**	**557**	**-3.7**
207542_s_at	aquaporin 1 (channel-forming integral protein, 28kDa)	AQP1	**4729**	**1273**	**-3.7**
203505_at	ATP-binding cassette, sub-family A (ABC1), member 1	ABCA1	**244**	**68**	**-3.6**
209821_at	chromosome 9 open reading frame 26 (NF-HEV)	C9orf26	**767**	**235**	**-3.3**
202661_at	inositol 1,4,5-triphosphate receptor, type 2	ITPR2	**330**	**108**	**-3.1**
210379_s_at	tousled-like kinase 1	TLK1	**166**	**55**	**-3.0**
222344_at	Chromosome 5 open reading frame 13	C5orf13	**345**	**116**	**-3.0**
209047_at	aquaporin 1 (channel-forming integral protein, 28kDa)	AQP1	**4709**	**1583**	**-3.0**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change –Control/Thrombin
41660_at	cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog, Drosophila)	CELSR1	**163**	**56**	**-2.9**
211466_at	nuclear factor I/B	NFIB	**246**	**88**	**-2.8**
201291_s_at	topoisomerase (DNA) II alpha 170kDa	TOP2A	**176**	**63**	**-2.8**
204015_s_at	dual specificity phosphatase 4	DUSP4	**350**	**125**	**-2.8**
221735_at	WD repeat domain 48	WDR48	**489**	**179**	**-2.7**
214787_at	c-myc promoter binding protein	MYCPBP	**234**	**87**	**-2.7**
212789_at	KIAA0056 protein	hCAP-D3	**217**	**82**	**-2.7**
205174_s_at	glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	QPCT	**217**	**84**	**-2.6**
209160_at	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	AKR1C3	**233**	**91**	**-2.6**
220735_s_at	SUMO1/sentrin specific peptidase 7	SENP7	**230**	**89**	**-2.6**
207761_s_at	DKFZP586A0522 protein	DKFZP586A 0522	**663**	**260**	**-2.5**
211789_s_at	MondoA	MONDOA	**280**	**110**	**-2.5**
209898_x_at	intersectin 2	ITSN2	**738**	**290**	**-2.5**
209735_at	ATP-binding cassette, sub-family G (WHITE), member 2	ABCG2	**3051**	**1207**	**-2.5**
206950_at	sodium channel, voltage-gated, type IX, alpha	SCN9A	**338**	**136**	**-2.5**
218918_at	mannosidase, alpha, class 1C, member 1	MAN1C1	**1881**	**756**	**-2.5**
214881_s_at	upstream binding transcription factor, RNA polymerase I	UBTF	**213**	**86**	**-2.5**
201767_s_at	elaC homolog 2 (E. coli)	ELAC2	**206**	**84**	**-2.4**
208935_s_at	lectin, galactoside-binding, soluble, 8 (galectin 8)	LGALS8	**330**	**137**	**-2.4**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change –Control/Thrombin
214717_at	hypothetical protein DKFZp434H1419	DKFZp434H1419	**268**	**113**	**-2.4**
209642_at	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	BUB1	**204**	**86**	**-2.4**
202660_at	Family with sequence similarity 20, member C	ITPR2	**485**	**204**	**-2.4**
204162_at	kinetochore associated 2	KNTC2	**160**	**67**	**-2.4**
202437_s_at	cytochrome P450, family 1, subfamily B, polypeptide 1	CYP1B1	**357**	**152**	**-2.4**
212418_at	E74-like factor 1 (ets domain transcription factor)	ELF1	**514**	**220**	**-2.3**
217703_x_at	Spondin 1, extracellular matrix protein	SPON1	**785**	**339**	**-2.3**
204071_s_at	topoisomerase I binding, arginine/serine-rich	TOPORS	**298**	**130**	**-2.3**
222145_at	hypothetical protein LOC440345	LOC440345	**219**	**96**	**-2.3**
206363_at	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	MAF	**457**	**200**	**-2.3**
205805_s_at	receptor tyrosine kinase-like orphan receptor 1	ROR1	**297**	**131**	**-2.3**
205749_at	cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	**607**	**269**	**-2.3**
202497_x_at	solute carrier family 2 (facilitated glucose transporter), member 3	SLC2A3	**808**	**358**	**-2.3**
205211_s_at	Ras and Rab interactor 1	RIN1	**252**	**112**	**-2.3**
210202_s_at	bridging integrator 1	BIN1	**463**	**208**	**-2.2**
214620_x_at	peptidylglycine alpha-amidating monooxygenase	PAM	**1795**	**806**	**-2.2**
212019_at	ribosomal L1 domain containing 1	RSL1D1	**192**	**87**	**-2.2**
218988_at	solute carrier family 35, member E3	SLC35E3	**316**	**143**	**-2.2**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change –Control/Thrombin
219166_at	chromosome 14 open reading frame 104	C14orf104	**270**	**123**	**-2.2**
213033_s_at	Nuclear factor I/B	NFIB	**874**	**402**	**-2.2**
37549_g_at	parathyroid hormone-responsive B1	PTHB1	**279**	**129**	**-2.2**
205961_s_at	PC4 and SFRS1 interacting protein 1	PSIP1	**639**	**297**	**-2.2**
221820_s_at	MYST histone acetyltransferase 1	MYST1	**196**	**91**	**-2.2**
215028_at	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	SEMA6A	**211**	**98**	**-2.1**
35179_at	beta-1,3-glucuronyltransferase 3 (glucuronosyltransferase I)	B3GAT3	**194**	**91**	**-2.1**
215447_at	n.a.		**789**	**371**	**-2.1**
218452_at	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a-like 1	SMARCAL1	**209**	**98**	**-2.1**
221031_s_at	hypothetical protein DKFZp434F0318	DKFZP434F0318	**1067**	**502**	**-2.1**
201309_x_at	chromosome 5 open reading frame 13	C5orf13	**3106**	**1464**	**-2.1**
219181_at	lipase, endothelial	LIPG	**492**	**232**	**-2.1**
204364_s_at	chromosome 2 open reading frame 23	C2orf23	**640**	**303**	**-2.1**
218585_s_at	denticleless homolog (Drosophila)	DTL	**271**	**130**	**-2.1**
202498_s_at	solute carrier family 2 (facilitated glucose transporter), member 3	SLC2A3	**189**	**90**	**-2.1**
213894_at	KIAA0960 protein	KIAA0960	**1049**	**502**	**-2.1**
202499_s_at	solute carrier family 2 (facilitated glucose transporter), member 3	SLC2A3	**336**	**161**	**-2.1**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change –Control/Thrombin
40687_at	gap junction protein, alpha 4, 37kDa (connexin 37)	GJA4	**1148**	**551**	**-2.1**
209936_at	RNA binding motif protein 5	RBM5	**246**	**118**	**-2.1**
203126_at	inositol(myo)-1(or 4)-monophosphatase 2	IMPA2	**157**	**76**	**-2.1**
212981_s_at	KIAA0738 gene product	KIAA0738	**181**	**88**	**-2.1**
201411_s_at	pleckstrin homology domain containing, family B (evectins) member 2	PLEKHB2	**515**	**249**	**-2.1**
205501_at	CDNA FLJ25677 fis, clone TST04054		**197**	**96**	**-2.1**
213146_at	jumonji domain containing 3	JMJD3	**257**	**125**	**-2.1**
208776_at	proteasome (prosome, macropain) 26S subunit, non-ATPase, 11	PSMD11	**272**	**133**	**-2.1**
205811_at	polymerase (DNA directed), gamma 2, accessory subunit	POLG2	**210**	**103**	**-2.0**
215248_at	growth factor receptor-bound protein 10	GRB10	**538**	**263**	**-2.0**
212192_at	potassium channel tetramerisation domain containing 12	KCTD12	**4208**	**2067**	**-2.0**
204681_s_at	Rap guanine nucleotide exchange factor (GEF) 5	RAPGEF5	**3047**	**1505**	**-2.0**
221196_x_at	chromosome X open reading frame 53	CXorf53	**434**	**214**	**-2.0**
202783_at	nicotinamide nucleotide transhydrogenase	NNT	**696**	**344**	**-2.0**
217527_s_at	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 interacting protein	NFATC2IP	**729**	**361**	**-2.0**
205960_at	pyruvate dehydrogenase kinase, isoenzyme 4	PDK4	**445**	**221**	**-2.0**
208763_s_at	TSC22 domain family, member 3	TSC22D3	**1015**	**503**	**-2.0**
219336_s_at	activating signal cointegrator 1 complex subunit 1	ASCC1	**309**	**153**	**-2.0**
209513_s_at	hydroxysteroid dehydrogenase like 2	HSDL2	**259**	**128**	**-2.0**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change –Control/Thrombin
203749_s_at	retinoic acid receptor, alpha	RARA	**475**	**236**	**-2.0**
204759_at	regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 2	RCBTB2	**646**	**321**	**-2.0**
212366_at	zinc finger protein 292	ZNF292	**205**	**102**	**-2.0**
205581_s_at	nitric oxide synthase 3 (endothelial cell)	NOS3	**423**	**211**	**-2.0**
208415_x_at	inhibitor of growth family, member 1	ING1	**518**	**258**	**-2.0**
205529_s_at	runt-related transcription factor 1; translocated to, 1 (cyclin D-related)	RUNX1T1	**412**	**206**	**-2.0**
204290_s_at	aldehyde dehydrogenase 6 family, member A1	ALDH6A1	**640**	**319**	**-2.0**
213463_s_at	KIAA0974	KIAA0974	**210**	**105**	**-2.0**

Table 13. Probe sets up-regulated (A) or down-regulated (B) >2-fold by thrombin at 24 h. Data were filtered according to the following criteria: detection *p*-value <0.065 and signal value >150 in either the control or the stimulated group; fold change >2.0. Results of one experiment are shown. n.a.: not annotated.

A) Probes sets up-regulated >2-fold by thrombin at 24 h

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change Thrombin/Control
206942_s_at	pro-melanin-concentrating hormone	PMCH	**100**	**1066**	**10.7**
210628_x_at	latent transforming growth factor beta binding protein 4	LTBP4	**25**	**196**	**7.7**
201242_s_at	ATPase, Na+/K+ transporting, beta 1 polypeptide	ATP1B1	**130**	**642**	**4.9**
220116_at	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	KCNN2	**118**	**537**	**4.5**
206775_at	cubilin (intrinsic factor-cobalamin receptor)	CUBN	**66**	**290**	**4.4**
205260_s_at	acylphosphatase 1, erythrocyte (common) type	ACYP1	**45**	**191**	**4.2**
204222_s_at	GLI pathogenesis-related 1 (glioma)	GLIPR1	**49**	**201**	**4.1**
204726_at	cadherin 13, H-cadherin (heart)	CDH13	**134**	**529**	**4.0**
211177_s_at	thioredoxin reductase 2	TXNRD2	**151**	**588**	**3.9**
201058_s_at	myosin, light polypeptide 9, regulatory	MYL9	**141**	**543**	**3.8**
204748_at	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	**52**	**192**	**3.7**
202859_x_at	interleukin 8	IL8	**224**	**818**	**3.6**
202291_s_at	matrix Gla protein	MGP	**3100**	**11265**	**3.6**
205659_at	histone deacetylase 9	HDAC9	**108**	**380**	**3.5**
206211_at	selectin E (endothelial adhesion molecule 1)	SELE	**75**	**264**	**3.5**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change Thrombin/Control
214085_x_at	GLI pathogenesis-related 1 (glioma)	GLIPR1	**129**	**437**	**3.4**
208581_x_at	metallothionein 1X	MT1X	**387**	**1311**	**3.4**
203213_at	Cell division cycle 2, G1 to S and G2 to M	CDC2	**282**	**907**	**3.2**
202627_s_at	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	SERPINE1	**3705**	**11860**	**3.2**
204340_at	chromosome X open reading frame 12	CXorf12	**74**	**238**	**3.2**
210764_s_at	cysteine-rich, angiogenic inducer, 61	CYR61	**1209**	**3781**	**3.1**
222155_s_at	G protein-coupled receptor 172A	GPR172A	**162**	**507**	**3.1**
208394_x_at	endothelial cell-specific molecule 1	ESM1	**441**	**1348**	**3.1**
217165_x_at	metallothionein 1F (functional)	MT1F	**186**	**542**	**2.9**
209373_at	mal, T-cell differentiation protein-like	MALL	**562**	**1626**	**2.9**
204887_s_at	polo-like kinase 4 (Drosophila)	PLK4	**123**	**343**	**2.8**
209277_at	Tissue factor pathway inhibitor 2	TFPI2	**842**	**2316**	**2.8**
208138_at	gastrin	GAST	**92**	**254**	**2.8**
210484_s_at	tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	TNFRSF10C	**158**	**426**	**2.7**
60794_f_at	zinc finger protein 587	ZNF587	**66**	**176**	**2.7**
221521_s_at	DNA replication complex GINS protein PSF2	Pfs2	**89**	**237**	**2.7**
201291_s_at	topoisomerase (DNA) II alpha 170kDa	TOP2A	**140**	**368**	**2.6**
219555_s_at	uncharacterized bone marrow protein BM039	BM039	**93**	**243**	**2.6**
202269_x_at	guanylate binding protein 1, interferon-inducible, 67kDa	GBP1	**69**	**180**	**2.6**
218368_s_at	tumor necrosis factor receptor superfamily, member 12A	TNFRSF12A	**312**	**800**	**2.6**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change Thrombin/Control
203917_at	coxsackie virus and adenovirus receptor	CXADR	**154**	**395**	**2.6**
201289_at	cysteine-rich, angiogenic inducer, 61	CYR61	**1843**	**4699**	**2.6**
204822_at	TTK protein kinase	TTK	**83**	**212**	**2.5**
203821_at	heparin-binding EGF-like growth factor	HBEGF	**311**	**784**	**2.5**
203362_s_at	MAD2 mitotic arrest deficient-like 1 (yeast)	MAD2L1	**185**	**466**	**2.5**
222266_at	Chromosome 19 open reading frame 2	C19orf2	**64**	**155**	**2.4**
202628_s_at	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	SERPINE1	**4033**	**9765**	**2.4**
221059_s_at	coactosin-like 1 (Dictyostelium)	COTL1	**305**	**736**	**2.4**
222206_s_at	nicalin homolog (zebrafish)	NCLN	**77**	**184**	**2.4**
209642_at	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	BUB1	**101**	**239**	**2.4**
215445_x_at	Clone 23605 mRNA sequence		**71**	**166**	**2.4**
201890_at	ribonucleotide reductase M2 polypeptide	RRM2	**355**	**831**	**2.3**
218663_at	chromosome condensation protein G	HCAP-G	**127**	**295**	**2.3**
218782_s_at	ATPase family, AAA domain containing 2	ATAD2	**143**	**329**	**2.3**
203868_s_at	vascular cell adhesion molecule 1	VCAM1	**119**	**273**	**2.3**
218992_at	chromosome 9 open reading frame 46	C9orf46	**142**	**326**	**2.3**
210457_x_at	high mobility group AT-hook 1	HMGA1	**336**	**768**	**2.3**
219918_s_at	asp (abnormal spindle)-like, microcephaly associated (Drosophila)	ASPM	**194**	**445**	**2.3**
204162_at	kinetochore associated 2	KNTC2	**112**	**256**	**2.3**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change Thrombin/Control
221723_s_at	solute carrier family 4, sodium bicarbonate cotransporter, member 5	SLC4A5	**92**	**209**	**2.3**
214734_at	exophilin 5	EXPH5	**95**	**216**	**2.3**
201243_s_at	ATPase, Na+/K+ transporting, beta 1 polypeptide	ATP1B1	**380**	**859**	**2.3**
211148_s_at	angiopoietin 2	ANGPT2	**2719**	**6068**	**2.2**
219978_s_at	nucleolar and spindle associated protein 1	NUSAP1	**198**	**439**	**2.2**
213454_at	apoptosis-inducing, TAF9-like domain 1	APITD1	**108**	**239**	**2.2**
206429_at	coagulation factor II (thrombin) receptor-like 1	F2RL1	**192**	**426**	**2.2**
210559_s_at	cell division cycle 2, G1 to S and G2 to M	CDC2	**574**	**1267**	**2.2**
212185_x_at	metallothionein 2A	MT2A	**1692**	**3727**	**2.2**
209891_at	spindle pole body component 25 homolog (S. cerevisiae)	SPBC25	**84**	**185**	**2.2**
205680_at	matrix metallopeptidase 10 (stromelysin 2)	MMP10	**547**	**1201**	**2.2**
206566_at	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	SLC7A1	**116**	**254**	**2.2**
204159_at	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	CDKN2C	**87**	**190**	**2.2**
219582_at	opioid growth factor receptor-like 1	OGFRL1	**233**	**507**	**2.2**
217998_at	pleckstrin homology-like domain, family A, member 1	PHLDA1	**565**	**1228**	**2.2**
222037_at	MCM4 minichromosome maintenance deficient 4 (S. cerevisiae)	MCM4	**108**	**234**	**2.2**
218935_at	EH-domain containing 3	EHD3	**469**	**1016**	**2.2**
213506_at	coagulation factor II (thrombin) receptor-like 1	F2RL1	**189**	**408**	**2.2**
211506_s_at	interleukin 8	IL8	**169**	**364**	**2.2**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change Thrombin/Control
211163_s_at	tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	TNFRSF10C	**421**	**907**	**2.2**
214126_at	Mitochondrial carrier triple repeat 1	MCART1	**71**	**151**	**2.1**
211685_s_at	neurocalcin delta	NCALD	**79**	**168**	**2.1**
202496_at	autoantigen	RCD-8	**204**	**434**	**2.1**
211456_x_at	Similar to 60S ribosomal protein L35	RPL35	**1038**	**2200**	**2.1**
222036_s_at	MCM4 minichromosome maintenance deficient 4 (S. cerevisiae)	MCM4	**279**	**590**	**2.1**
201431_s_at	dihydropyrimidinase-like 3	DPYSL3	**595**	**1256**	**2.1**
48808_at	dihydrofolate reductase	DHFR	**185**	**390**	**2.1**
218542_at	chromosome 10 open reading frame 3	C10orf3	**249**	**524**	**2.1**
200629_at	tryptophanyl-tRNA synthetase	WARS	**1722**	**3626**	**2.1**
214040_s_at	gelsolin (amyloidosis, Finnish type)	GSN	**406**	**852**	**2.1**
32069_at	Nedd4 binding protein 1	N4BP1	**139**	**291**	**2.1**
204200_s_at	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	PDGFB	**454**	**951**	**2.1**
204146_at	RAD51 associated protein 1	RAD51AP1	**74**	**154**	**2.1**
210145_at	phospholipase A2, group IVA (cytosolic, calcium-dependent)	PLA2G4A	**273**	**567**	**2.1**
210052_s_at	TPX2, microtubule-associated, homolog (Xenopus laevis)	TPX2	**512**	**1062**	**2.1**
202422_s_at	acyl-CoA synthetase long-chain family member 4	ACSL4	**218**	**452**	**2.1**
205572_at	angiopoietin 2	ANGPT2	**2255**	**4656**	**2.1**
203235_at	thimet oligopeptidase 1	THOP1	**91**	**187**	**2.1**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change Thrombin/Control
205973_at	fasciculation and elongation protein zeta 1 (zygin I)	FEZ1	**161**	**330**	**2.1**
210298_x_at	four and a half LIM domains 1	FHL1	**868**	**1775**	**2.0**
218887_at	mitochondrial ribosomal protein L2	MRPL2	**251**	**509**	**2.0**
209369_at	annexin A3	ANXA3	**407**	**825**	**2.0**
210517_s_at	A kinase (PRKA) anchor protein (gravin) 12	AKAP12	**4040**	**8127**	**2.0**

B) Probe sets down-regulated >2-fold by thrombin at 24 h

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change –Control/Thrombin
217588_at	cation channel, sperm associated 2	CATSPER2	**174**	**17**	**-10.0**
207542_s_at	aquaporin 1 (channel-forming integral protein, 28kDa)	AQP1	**4492**	**778**	**-5.8**
209047_at	aquaporin 1 (channel-forming integral protein, 28kDa)	AQP1	**4757**	**975**	**-4.9**
222278_at	hypothetical LOC389393	LOC389393	**272**	**71**	**-3.8**
221735_at	WD repeat domain 48	WDR48	**459**	**123**	**-3.7**
219935_at	ADAM metallopeptidase with thrombospondin type 1 motif, 5 (aggrecanase-2)	ADAMTS5	**606**	**165**	**-3.7**
202437_s_at	cytochrome P450, family 1, subfamily B, polypeptide 1	CYP1B1	**349**	**96**	**-3.6**
209469_at	glycoprotein M6A	GPM6A	**161**	**47**	**-3.4**
207735_at	ring finger protein 125	RNF125	**172**	**52**	**-3.3**
221031_s_at	hypothetical protein DKFZp434F0318	DKFZP434F0318	**1261**	**380**	**-3.3**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change –Control/Thrombin
205020_s_at	ADP-ribosylation factor-like 4	ARL4	**1882**	**583**	**-3.2**
209470_s_at	glycoprotein M6A	GPM6A	**236**	**76**	**-3.1**
210025_s_at	caspase recruitment domain family, member 10	CARD10	**156**	**53**	**-3.0**
202478_at	tribbles homolog 2 (Drosophila)	TRIB2	**689**	**238**	**-2.9**
202661_at	inositol 1,4,5-triphosphate receptor, type 2	ITPR2	**353**	**123**	**-2.9**
206638_at	5-hydroxytryptamine (serotonin) receptor 2B	HTR2B	**459**	**162**	**-2.8**
203973_s_at	CCAAT/enhancer binding protein (C/EBP), delta	CEBPD	**440**	**158**	**-2.8**
204596_s_at	stanniocalcin 1	STC1	**1228**	**443**	**-2.8**
205749_at	cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	**603**	**218**	**-2.8**
218918_at	mannosidase, alpha, class 1C, member 1	MAN1C1	**2194**	**796**	**-2.8**
204620_s_at	chondroitin sulfate proteoglycan 2 (versican)	CSPG2	**408**	**149**	**-2.7**
212188_at	potassium channel tetramerisation domain containing 12	KCTD12	**2793**	**1046**	**-2.7**
211212_s_at	origin recognition complex, subunit 5-like (yeast)	ORC5L	**194**	**74**	**-2.6**
210665_at	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	TFPI	**1090**	**416**	**-2.6**
221731_x_at	chondroitin sulfate proteoglycan 2 (versican)	CSPG2	**421**	**161**	**-2.6**
219371_s_at	Kruppel-like factor 2 (lung)	KLF2	**807**	**312**	**-2.6**
216882_s_at	nebulette	NEBL	**210**	**82**	**-2.6**
201309_x_at	chromosome 5 open reading frame 13	C5orf13	**3228**	**1259**	**-2.6**
206950_at	sodium channel, voltage-gated, type IX, alpha	SCN9A	**350**	**138**	**-2.5**
209347_s_at	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	MAF	**247**	**99**	**-2.5**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change –Control/Thrombin
214417_s_at	Fetuin B	FETUB	**215**	**86**	**-2.5**
209505_at	Nuclear receptor subfamily 2, group F, member 1	NR2F1	**309**	**124**	**-2.5**
204595_s_at	stanniocalcin 1	STC1	**2407**	**986**	**-2.4**
218559_s_at	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	MAFB	**795**	**330**	**-2.4**
221009_s_at	angiopoietin-like 4	ANGPTL4	**208**	**87**	**-2.4**
208033_s_at	AT-binding transcription factor 1	ATBF1	**232**	**98**	**-2.4**
214449_s_at	ras homolog gene family, member Q	RHOQ	**215**	**91**	**-2.4**
205392_s_at	chemokine (C-C motif) ligand 14	CCL14	**8409**	**3618**	**-2.3**
218205_s_at	MAP kinase interacting serine/threonine kinase 2	MKNK2	**4176**	**1828**	**-2.3**
204597_x_at	stanniocalcin 1	STC1	**2655**	**1172**	**-2.3**
205346_at	ST3 beta-galactoside alpha-2,3-sialyltransferase 2	ST3GAL2	**165**	**74**	**-2.2**
41660_at	cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog, Drosophila)	CELSR1	**172**	**77**	**-2.2**
212192_at	potassium channel tetramerisation domain containing 12	KCTD12	**4096**	**1829**	**-2.2**
204681_s_at	Rap guanine nucleotide exchange factor (GEF) 5	RAPGEF5	**3628**	**1630**	**-2.2**
209676_at	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	TFPI	**4254**	**1920**	**-2.2**
201310_s_at	chromosome 5 open reading frame 13	C5orf13	**7513**	**3401**	**-2.2**
206363_at	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	MAF	**454**	**206**	**-2.2**
218819_at	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26	DDX26	**232**	**105**	**-2.2**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change –Control/Thrombin
212419_at	chromosome 10 open reading frame 56	C10orf56	**960**	**437**	**-2.2**
214156_at	myosin VIIA and Rab interacting protein	MYRIP	**152**	**69**	**-2.2**
219488_at	alpha 1,4-galactosyltransferase (globotriaosylceramide synthase)	A4GALT	**197**	**91**	**-2.2**
203886_s_at	fibulin 2	FBLN2	**473**	**218**	**-2.2**
216100_s_at	torsin A interacting protein 1	TOR1AIP1	**342**	**159**	**-2.2**
209730_at	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F	SEMA3F	**440**	**204**	**-2.2**
209105_at	nuclear receptor coactivator 1	NCOA1	**338**	**158**	**-2.1**
202723_s_at	forkhead box O1A (rhabdomyosarcoma)	FOXO1A	**1641**	**781**	**-2.1**
204359_at	fibronectin leucine rich transmembrane protein 2	FLRT2	**3589**	**1709**	**-2.1**
203225_s_at	riboflavin kinase	RFK	**2516**	**1200**	**-2.1**
219880_at	n.a.	n.a.	**206**	**99**	**-2.1**
204949_at	intercellular adhesion molecule 3	ICAM3	**192**	**92**	**-2.1**
209821_at	chromosome 9 open reading frame 26 (NF-HEV)	C9orf26	**584**	**281**	**-2.1**
214035_x_at	LOC399491 protein	LOC399491	**1794**	**864**	**-2.1**
212224_at	aldehyde dehydrogenase 1 family, member A1	ALDH1A1	**4625**	**2242**	**-2.1**
209348_s_at	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	MAF	**997**	**483**	**-2.1**
205960_at	pyruvate dehydrogenase kinase, isoenzyme 4	PDK4	**411**	**203**	**-2.0**

Affymetrix probe set ID	Gene title	Gene symbol	Control	Thrombin	Fold change –Control/Thrombin
203551_s_at	COX11 homolog, cytochrome c oxidase assembly protein (yeast)	COX11	**348**	**172**	**-2.0**
213258_at	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	TFPI	**7502**	**3723**	**-2.0**

Table 14. Probe sets up-regulated (A) or down-regulated (B) >2-fold by LTD_4 plus thrombin at 1 h. Data were filtered according to the following criteria: detection *p*-value <0.065 at least in two of three samples in either the control or the stimulated group; signal value >150 at least in two of three samples in either the control or the stimulated group; fold change calculated from the signal means >2.0. Mean signals of three experiments are shown. n.a.: not annotated

A) Probe sets up-regulated >2-fold by LTD_4 plus thrombin at 1 h

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4 + Thrombin	Fold change Control/LTD_4 + Thrombin
202768_at	FBJ murine osteosarcoma viral oncogene homolog B	FOSB	**27**	**5351**	**196.3**
216248_s_at	nuclear receptor subfamily 4, group A, member 2	NR4A2	**52**	**6240**	**119.9**
204621_s_at	nuclear receptor subfamily 4, group A, member 2	NR4A2	**28**	**3247**	**114.1**
209189_at	v-fos FBJ murine osteosarcoma viral oncogene homolog	FOS	**23**	**2098**	**90.1**
204622_x_at	nuclear receptor subfamily 4, group A, member 2	NR4A2	**93**	**5306**	**57.1**
206115_at	early growth response 3	EGR3	**95**	**4178**	**44.0**
202340_x_at	nuclear receptor subfamily 4, group A, member 1	NR4A1	**105**	**4232**	**40.2**
209959_at	nuclear receptor subfamily 4, group A, member 3	NR4A3	**37**	**1454**	**38.5**
202859_x_at	interleukin 8	IL8	**235**	**6052**	**25.7**
210764_s_at	cysteine-rich, angiogenic inducer, 61	CYR61	**784**	**18409**	**23.5**
204363_at	coagulation factor III (thromboplastin, tissue factor)	F3	**25**	**592**	**23.0**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4 + Thrombin	Fold change Control/LTD_4 + Thrombin
204748_at	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	**344**	**7043**	**20.5**
201044_x_at	dual specificity phosphatase 1	DUSP1	**37**	**738**	**19.9**
203821_at	heparin-binding EGF-like growth factor	HBEGF	**273**	**5355**	**19.6**
208370_s_at	Down syndrome critical region gene 1	DSCR1	**412**	**7836**	**19.0**
202672_s_at	Activating transcription factor 3	ATF3	**283**	**5091**	**17.9**
207978_s_at	nuclear receptor subfamily 4, group A, member 3	NR4A3	**111**	**1882**	**16.9**
205249_at	early growth response 2 (Krox-20 homolog, Drosophila)	EGR2	**98**	**1634**	**16.5**
201531_at	zinc finger protein 36, C3H type, homolog (mouse)	ZFP36	**286**	**4517**	**15.8**
201289_at	cysteine-rich, angiogenic inducer, 61	CYR61	**919**	**12231**	**13.3**
222162_s_at	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	ADAMTS1	**593**	**6967**	**11.7**
211506_s_at	interleukin 8	IL8	**182**	**1982**	**10.9**
209774_x_at	chemokine (C-X-C motif) ligand 2	CXCL2	**318**	**3235**	**10.2**
38037_at	heparin-binding EGF-like growth factor	HBEGF	**214**	**1988**	**9.3**
208078_s_at	SNF1-like kinase	SNF1LK	**377**	**3287**	**8.7**
215254_at	Down syndrome critical region gene 1	DSCR1	**96**	**806**	**8.4**
218541_s_at	chromosome 8 open reading frame 4	C8orf4	**129**	**1079**	**8.4**
202861_at	period homolog 1 (Drosophila)	PER1	**93**	**767**	**8.2**
215253_s_at	Down syndrome critical region gene 1	DSCR1	**509**	**4158**	**8.2**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4 + Thrombin	Fold change Control/LTD_4 + Thrombin
221841_s_at	Kruppel-like factor 4 (gut)	KLF4	**484**	**3852**	**8.0**
201041_s_at	dual specificity phosphatase 1	DUSP1	**1786**	**13186**	**7.4**
221031_s_at	hypothetical protein DKFZp434F0318	DKFZP434F0318	**549**	**3766**	**6.8**
220266_s_at	Kruppel-like factor 4 (gut)	KLF4	**227**	**1500**	**6.6**
204222_s_at	GLI pathogenesis-related 1 (glioma)	GLIPR1	**58**	**384**	**6.6**
201694_s_at	early growth response 1	EGR1	**593**	**3606**	**6.1**
205027_s_at	mitogen-activated protein kinase kinase kinase 8	MAP3K8	**32**	**186**	**5.7**
200796_s_at	myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	**374**	**2112**	**5.6**
200797_s_at	myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	**1950**	**10979**	**5.6**
204472_at	GTP binding protein overexpressed in skeletal muscle	GEM	**176**	**989**	**5.6**
204135_at	downregulated in ovarian cancer 1	DOC1	**539**	**2985**	**5.5**
202150_s_at	neural precursor cell expressed, developmentally down-regulated 9	NEDD9	**571**	**2985**	**5.2**
212614_at	AT rich interactive domain 5B (MRF1-like)	ARID5B	**485**	**2507**	**5.2**
202388_at	regulator of G-protein signalling 2, 24kDa	RGS2	**424**	**2098**	**4.9**
203068_at	kelch-like 21 (Drosophila)	KLHL21	**113**	**556**	**4.9**
218880_at	FOS-like antigen 2	FOSL2	**228**	**1110**	**4.9**
209967_s_at	cAMP responsive element modulator	CREM	**299**	**1426**	**4.8**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4 + Thrombin	Fold change Control/LTD_4 + Thrombin
202149_at	neural precursor cell expressed, developmentally down-regulated 9	NEDD9	**532**	**2520**	**4.7**
210511_s_at	inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	**128**	**606**	**4.7**
200798_x_at	myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	**1559**	**7289**	**4.7**
215199_at	caldesmon 1	CALD1	**114**	**493**	**4.3**
208960_s_at	Kruppel-like factor 6	KLF6	**810**	**3474**	**4.3**
214438_at	H2.0-like homeo box 1 (Drosophila)	HLX1	**516**	**2206**	**4.3**
215198_s_at	caldesmon 1	CALD1	**146**	**615**	**4.2**
214446_at	elongation factor, RNA polymerase II, 2	ELL2	**89**	**367**	**4.1**
206765_at	potassium inwardly-rectifying channel, subfamily J, member 2	KCNJ2	**751**	**3076**	**4.1**
209357_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	CITED2	**309**	**1239**	**4.0**
208961_s_at	Kruppel-like factor 6	KLF6	**1517**	**5769**	**3.8**
201739_at	serum/glucocorticoid regulated kinase	SGK	**1173**	**4388**	**3.7**
204011_at	sprouty homolog 2 (Drosophila)	SPRY2	**500**	**1846**	**3.7**
208415_x_at	inhibitor of growth family, member 1	ING1	**168**	**620**	**3.7**
201693_s_at	early growth response 1	EGR1	**287**	**1048**	**3.6**
205290_s_at	bone morphogenetic protein 2	BMP2	**1299**	**4728**	**3.6**
219371_s_at	Kruppel-like factor 2 (lung)	KLF2	**1140**	**3996**	**3.5**
213684_s_at	PDZ and LIM domain 5	PDLIM5	**45**	**157**	**3.5**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4 + Thrombin	Fold change Control/LTD_4 + Thrombin
202270_at	guanylate binding protein 1, interferon-inducible, 67kDa	GBP1	**54**	**183**	**3.4**
209101_at	connective tissue growth factor	CTGF	**5002**	**16776**	**3.4**
217996_at	pleckstrin homology-like domain, family A, member 1	PHLDA1	**744**	**2439**	**3.3**
205289_at	bone morphogenetic protein 2	BMP2	**1063**	**3450**	**3.2**
204470_at	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	CXCL1	**141**	**457**	**3.2**
200632_s_at	N-myc downstream regulated gene 1	NDRG1	**1363**	**4367**	**3.2**
205409_at	FOS-like antigen 2	FOSL2	**126**	**404**	**3.2**
207980_s_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	CITED2	**345**	**1100**	**3.2**
201466_s_at	v-jun sarcoma virus 17 oncogene homolog (avian)	JUN	**307**	**978**	**3.2**
207850_at	chemokine (C-X-C motif) ligand 3	CXCL3	**51**	**162**	**3.2**
203751_x_at	jun D proto-oncogene	JUND	**392**	**1223**	**3.1**
217997_at	pleckstrin homology-like domain, family A, member 1	PHLDA1	**431**	**1344**	**3.1**
218881_s_at	FOS-like antigen 2	FOSL2	**204**	**618**	**3.0**
214085_x_at	GLI pathogenesis-related 1 (glioma)	GLIPR1	**334**	**1011**	**3.0**
202269_x_at	guanylate binding protein 1, interferon-inducible, 67kDa	GBP1	**210**	**635**	**3.0**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4 + Thrombin	Fold change Control/LTD_4 + Thrombin
212665_at	TCDD-inducible poly(ADP-ribose) polymerase	TIPARP	**553**	**1643**	**3.0**
203140_at	B-cell CLL/lymphoma 6 (zinc finger protein 51)	BCL6	**425**	**1243**	**2.9**
203574_at	nuclear factor, interleukin 3 regulated	NFIL3	**406**	**1174**	**2.9**
202241_at	tribbles homolog 1 (Drosophila)	TRIB1	**798**	**2274**	**2.9**
212418_at	E74-like factor 1 (ets domain transcription factor)	ELF1	**550**	**1558**	**2.8**
202067_s_at	low density lipoprotein receptor (familial hypercholesterolemia)	LDLR	**171**	**478**	**2.8**
207630_s_at	cAMP responsive element modulator	CREM	**868**	**2417**	**2.8**
214056_at	Myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	**163**	**447**	**2.7**
209074_s_at	TU3A protein	TU3A	**98**	**268**	**2.7**
218000_s_at	pleckstrin homology-like domain, family A, member 1	PHLDA1	**226**	**607**	**2.7**
213895_at	Epithelial membrane protein 1	EMP1	**318**	**848**	**2.7**
202014_at	protein phosphatase 1, regulatory (inhibitor) subunit 15A	PPP1R15A	**407**	**1066**	**2.6**
212558_at	sprouty homolog 1, antagonist of FGF signaling (Drosophila)	SPRY1	**637**	**1631**	**2.6**
36711_at	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	MAFF	**1676**	**4259**	**2.5**
208119_s_at	zinc finger protein 505	ZNF505	**126**	**320**	**2.5**
209457_at	dual specificity phosphatase 5	DUSP5	**1239**	**2995**	**2.4**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4 + Thrombin	Fold change Control/LTD_4 + Thrombin
220990_s_at	likely ortholog of rat vacuole membrane protein 1	VMP1	**1216**	**2921**	**2.4**
207526_s_at	interleukin 1 receptor-like 1	IL1RL1	**477**	**1144**	**2.4**
214508_x_at	cAMP responsive element modulator	CREM	**546**	**1305**	**2.4**
217998_at	pleckstrin homology-like domain, family A, member 1	PHLDA1	**411**	**982**	**2.4**
213146_at	jumonji domain containing 3	JMJD3	**179**	**421**	**2.4**
221763_at	jumonji domain containing 1C	JMJD1C	**768**	**1784**	**2.3**
203234_at	uridine phosphorylase 1	UPP1	**241**	**559**	**2.3**
217168_s_at	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	HERPUD1	**1523**	**3494**	**2.3**
37028_at	protein phosphatase 1, regulatory (inhibitor) subunit 15A	PPP1R15A	**729**	**1664**	**2.3**
215206_at	Exostoses (multiple) 1	EXT1	**226**	**511**	**2.3**
201473_at	jun B proto-oncogene	JUNB	**781**	**1765**	**2.3**
201464_x_at	v-jun sarcoma virus 17 oncogene homolog (avian)	JUN	**1614**	**3603**	**2.2**
204094_s_at	TSC22 domain family 2	TSC22D2	**552**	**1220**	**2.2**
202068_s_at	low density lipoprotein receptor (familial hypercholesterolemia)	LDLR	**1516**	**3321**	**2.2**
221489_s_at	sprouty homolog 4 (Drosophila)	SPRY4	**635**	**1380**	**2.2**
201925_s_at	decay accelerating factor for complement (CD55, Cromer blood group system)	DAF	**831**	**1787**	**2.2**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4 + Thrombin	Fold change Control/LTD_4 + Thrombin
204221_x_at	GLI pathogenesis-related 1 (glioma)	GLIPR1	**155**	**330**	**2.1**
202843_at	DnaJ (Hsp40) homolog, subfamily B, member 9	DNAJB9	**76**	**160**	**2.1**
207029_at	KIT ligand	KITLG	**157**	**332**	**2.1**
212724_at	Rho family GTPase 3	RND3	**2029**	**4261**	**2.1**
215990_s_at	B-cell CLL/lymphoma 6 (zinc finger protein 51)	BCL6	**393**	**823**	**2.1**
214038_at	chemokine (C-C motif) ligand 8	CCL8	**123**	**257**	**2.1**
201236_s_at	BTG family, member 2	BTG2	**1022**	**2137**	**2.1**
204093_at	cyclin H	CCNH	**836**	**1746**	**2.1**
202237_at	nicotinamide N-methyltransferase	NNMT	**815**	**1696**	**2.1**
201325_s_at	Epithelial membrane protein 1	EMP1	**1663**	**3443**	**2.1**
203543_s_at	Kruppel-like factor 9	KLF9	**367**	**756**	**2.1**
210762_s_at	deleted in liver cancer 1	DLC1	**1249**	**2556**	**2.0**
218995_s_at	endothelin 1	EDN1	**1493**	**3038**	**2.0**
201169_s_at	basic helix-loop-helix domain containing, class B, 2	BHLHB2	**415**	**836**	**2.0**
212420_at	E74-like factor 1 (ets domain transcription factor)	ELF1	**639**	**1285**	**2.0**
202499_s_at	solute carrier family 2 (facilitated glucose transporter), member 3	SLC2A3	**748**	**1504**	**2.0**
216979_at	nuclear receptor subfamily 4, group A, member 3	NR4A3	**92**	**185**	**2.0**
203542_s_at	Kruppel-like factor 9	KLF9	**609**	**1222**	**2.0**
204596_s_at	stanniocalcin 1	STC1	**1199**	**2405**	**2.0**

B) Probe sets down-regulated >2-fold by LTD_4 plus thrombin at 1 h

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4 + Thrombin	Fold change ─LTD_4 + Thrombin/Control
208937_s_at	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	ID1	**3144**	**831**	**-3.8**
201008_s_at	thioredoxin interacting protein	TXNIP	**2213**	**632**	**-3.5**
201009_s_at	thioredoxin interacting protein	TXNIP	**2952**	**867**	**-3.4**
201010_s_at	thioredoxin interacting protein	TXNIP	**3726**	**1142**	**-3.3**
203394_s_at	hairy and enhancer of split 1, (Drosophila)	HES1	**1345**	**430**	**-3.1**
202887_s_at	DNA-damage-inducible transcript 4	DDIT4	**1882**	**604**	**-3.1**
203395_s_at	hairy and enhancer of split 1, (Drosophila)	HES1	**1078**	**414**	**-2.6**
204790_at	SMAD, mothers against DPP homolog 7 (Drosophila)	SMAD7	**366**	**141**	**-2.6**
222303_at	n.a.		**320**	**132**	**-2.4**
201367_s_at	zinc finger protein 36, C3H type-like 2	ZFP36L2	**910**	**377**	**-2.4**
201369_s_at	zinc finger protein 36, C3H type-like 2	ZFP36L2	**692**	**301**	**-2.3**
207474_at	SNF related kinase	SNRK	**203**	**91**	**-2.2**
209905_at	homeo box A9	HOXA9	**438**	**198**	**-2.2**
217164_at	n.a.		**234**	**107**	**-2.2**
209602_s_at	GATA binding protein 3	GATA3	**211**	**96**	**-2.2**
209604_s_at	GATA binding protein 3	GATA3	**2286**	**1092**	**-2.1**
201939_at	polo-like kinase 2 (Drosophila)	PLK2	**1515**	**724**	**-2.1**
201368_at	zinc finger protein 36, C3H type-like 2	ZFP36L2	**4446**	**2165**	**-2.1**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4 + Thrombin	Fold change −LTD_4 + Thrombin/Control
214295_at	KIAA0485 protein	KIAA0485	**305**	**149**	**-2.0**
205020_s_at	ADP-ribosylation factor-like 4A	ARL4A	**1989**	**977**	**-2.0**
205955_at	n.a.		**165**	**82**	**-2.0**

Table 15. Probe sets up-regulated by LTD_4 plus thrombin and LTD_4 or thrombin. Mean signals of three experiments are shown.

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4	Thrombin	LTD_4 + Thrombin
38037_at	heparin-binding EGF-like growth factor	HBEGF	**214**	**967**	**1498**	**1988**
200632_s_at	N-myc downstream regulated gene 1	NDRG1	**1364**	**4032**	**3357**	**4367**
200797_s_at	myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	**1950**	**6556**	**9611**	**10979**
201041_s_at	dual specificity phosphatase 1	DUSP1	**1786**	**8797**	**8062**	**13187**
201044_x_at	dual specificity phosphatase 1	DUSP1	**37**	**433**	**277**	**738**
201289_at	cysteine-rich, angiogenic inducer, 61	CYR61	**920**	**7490**	**10516**	**12231**
201531_at	zinc finger protein 36, C3H type, homolog (mouse)	ZFP36	**287**	**3309**	**2367**	**4517**
201694_s_at	early growth response 1	EGR1	**594**	**1868**	**2608**	**3607**
202149_at	neural precursor cell expressed, developmentally down-regulated 9	NEDD9	**532**	**1995**	**2348**	**2520**
202150_s_at	neural precursor cell expressed, developmentally down-regulated 9	NEDD9	**571**	**1965**	**2391**	**2985**
202340_x_at	nuclear receptor subfamily 4, group A, member 1	NR4A1	**105**	**3363**	**2431**	**4233**
202388_at	Regulator of G-protein signalling 2, 24kDa	RGS2	**424**	**1994**	**1171**	**2099**
202672_s_at	activating transcription factor 3	ATF3	**284**	**1223**	**2437**	**5091**
202859_x_at	interleukin 8	IL8	**236**	**2080**	**3728**	**6053**
203821_at	heparin-binding EGF-like growth factor	HBEGF	**273**	**2598**	**3558**	**5355**
204363_at	coagulation factor III (thromboplastin, tissue factor)	F3	**26**	**400**	**177**	**592**
204472_at	GTP binding protein overexpressed in skeletal muscle	GEM	**177**	**523**	**486**	**990**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4	Thrombin	LTD_4 + Thrombin
204621_s_at	nuclear receptor subfamily 4, group A, member 2	NR4A2	**28**	**2904**	**1771**	**3248**
204622_x_at	nuclear receptor subfamily 4, group A, member 2	NR4A2	**93**	**4379**	**2855**	**5307**
204748_at	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	**344**	**5022**	**2602**	**7044**
205027_s_at	mitogen-activated protein kinase kinase kinase 8	MAP3K8	**33**	**159**	**83**	**186**
205249_at	early growth response 2 (Krox-20 homolog, Drosophila)	EGR2	**99**	**1012**	**739**	**1634**
205290_s_at	bone morphogenetic protein 2	BMP2	**1299**	**4345**	**2382**	**4728**
205960_at	Pyruvate dehydrogenase kinase, isoenzyme 4	PDK4	**441**	**1167**	**532**	**869**
206115_at	early growth response 3	EGR3	**95**	**2344**	**1856**	**4178**
206211_at	selectin E (endothelial adhesion molecule 1)	SELE	**47**	**159**	**83**	**148**
206765_at	potassium inwardly-rectifying channel, subfamily J, member 2	KCNJ2	**751**	**2638**	**1973**	**3077**
207978_s_at	nuclear receptor subfamily 4, group A, member 3	NR4A3	**112**	**1077**	**981**	**1883**
207980_s_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	CITED2	**345**	**807**	**1032**	**1100**
208078_s_at	SNF1-like kinase	SNF1LK	**377**	**2542**	**2659**	**3287**
208370_s_at	Down syndrome critical region gene 1	DSCR1	**413**	**6414**	**3096**	**7837**
209357_at	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	CITED2	**310**	**905**	**966**	**1239**
209774_x_at	chemokine (C-X-C motif) ligand 2	CXCL2	**318**	**2116**	**698**	**3236**
209959_at	nuclear receptor subfamily 4, group A, member 3	NR4A3	**38**	**878**	**815**	**1454**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4	Thrombin	LTD_4 + Thrombin
209967_s_at	cAMP responsive element modulator	CREM	**300**	**1528**	**994**	**1426**
210764_s_at	cysteine-rich, angiogenic inducer, 61	CYR61	**784**	**9244**	**14395**	**18409**
211506_s_at	interleukin 8	IL8	**182**	**637**	**1191**	**1982**
212614_at	AT rich interactive domain 5B (MRF1-like)	ARID5B	**485**	**1918**	**1849**	**2508**
214438_at	H2-like homeo box 1 (Drosophila)	HLX1	**517**	**2322**	**1685**	**2207**
214446_at	elongation factor, RNA polymerase II, 2	ELL2	**89**	**292**	**221**	**368**
214508_x_at	cAMP responsive element modulator	CREM	**546**	**1310**	**1022**	**1305**
215253_s_at	Down syndrome critical region gene 1	DSCR1	**510**	**3551**	**1454**	**4158**
216248_s_at	nuclear receptor subfamily 4, group A, member 2	NR4A2	**52**	**5338**	**3317**	**6241**
218880_at	FOS-like antigen 2	FOSL2	**228**	**747**	**760**	**1111**
219371_s_at	Kruppel-like factor 2 (lung)	KLF2	**1141**	**3013**	**3264**	**3997**
221031_s_at	hypothetical protein DKFZp434F0318	DKFZP434F0318	**550**	**2653**	**2209**	**3766**
221841_s_at	Kruppel-like factor 4 (gut)	KLF4	**484**	**1954**	**3263**	**3853**
222162_s_at	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	ADAMTS1	**594**	**5844**	**4240**	**6967**
200796_s_at	myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	**375**	**1105**	**1667**	**2113**
200798_x_at	myeloid cell leukemia sequence 1 (BCL2-related)	MCL1	**1560**	**4435**	**6164**	**7289**
201465_s_at	v-jun sarcoma virus 17 oncogene homolog (avian)	JUN	**169**	**242**	**480**	**644**
201466_s_at	v-jun sarcoma virus 17 oncogene homolog (avian)	JUN	**307**	**341**	**1221**	**978**
201739_at	serum/glucocorticoid regulated kinase	SGK	**1173**	**2213**	**3521**	**4388**
202269_x_at	guanylate binding protein 1, interferon-inducible, 67kDa	GBP1	**211**	**212**	**614**	**635**

Affymetrix probe set ID	Gene title	Gene symbol	Control	LTD_4	Thrombin	LTD_4 + Thrombin
202270_at	guanylate binding protein 1, interferon-inducible, 67kDa	GBP1	**54**	**90**	**212**	**184**
202768_at	FBJ murine osteosarcoma viral oncogene homolog B	FOSB	**27**	**710**	**4082**	**5352**
204011_at	sprouty homolog 2 (Drosophila)	SPRY2	**500**	**1292**	**1484**	**1846**
204135_at	downregulated in ovarian cancer 1	DOC1	**539**	**1309**	**2469**	**2986**
204222_s_at	GLI pathogenesis-related 1 (glioma)	GLIPR1	**58**	**105**	**270**	**384**
204470_at	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	CXCL1	**141**	**245**	**370**	**458**
208119_s_at	zinc finger protein 505	ZNF505	**127**	**197**	**359**	**321**
208960_s_at	Kruppel-like factor 6	KLF6	**811**	**1901**	**2914**	**3475**
208961_s_at	Kruppel-like factor 6	KLF6	**1518**	**3089**	**5006**	**5769**
209101_at	connective tissue growth factor	CTGF	**5003**	**8668**	**16656**	**16777**
214085_x_at	GLI pathogenesis-related 1 (glioma)	GLIPR1	**335**	**375**	**856**	**1011**
215198_s_at	caldesmon 1	CALD1	**147**	**264**	**557**	**616**
215199_at	caldesmon 1	CALD1	**115**	**224**	**393**	**494**
218541_s_at	chromosome 8 open reading frame 4	C8orf4	**129**	**402**	**660**	**1079**

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Publikationsliste

A) Zeitschriften

1. Uzonyi B, Lötzer K, Jahn S, Kramer C, Hildner M, Bretschneider E, Radke D, Beer M, Vollandt R, Evans JF, Funk CD, Habenicht AJ. Cysteinyl leukotriene 2 receptor and protease-activated receptor 1 activate strongly correlated early genes in human endothelial cells. Proc Natl Acad Sci U S A. 2006, 103:6326-6331

2. Bretschneider E, Uzonyi B, Weber AA, Fischer JW, Pape R, Lötzer K, Schrör K. Human Vascular Smooth Muscle Cells Express Functionally Active Endothelial Cell Protein C Receptor. Circulation Research. Epub 2006 Dec 14.

B) Veröffentlichte Konferenzbeiträge

1. N. John, K. Lötzer, B. Uzonyi, S. Jahn, C. Kramer, B. Kaiser, A. J. R. Habenicht: 5 Lipoxygenase und Atherosklerose. in: H. Heinle, H. Schulte, A. von Eckardstein: Stoffwechsel und Modifikation von Lipiden und Lipoproteinen (Kongressband von der 19. Jahrestagung der Deutschen Gesellschaft für Arterioskleroseforschung), 2005

Folgende der oben genannten Publikationen ist aus meiner Dissertation hervorgegangen:
A1

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Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe. Die Arbeit wurde bisher weder im Deutschland noch im Ausland in gleicher oder ähnlicher Form einem anderen Prüfungsamt vorgelegt.

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